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XXIII. THE EFFECT OF COLD STORAGE ON THE CARNOSINE CONTENT OF MUSCLE.

By WINIFRED MARY CLIFFORD.

From the Physiology Department, Household and Social Science Department, Kensington.

(*Received February 1st, 1922.*)

The flavour of meat has long been believed to be associated with the presence of extractives, although whenever isolated in a pure condition such substances are tasteless. However one extractive—β-alanyl-histidine or carnosine—does disappear in cold storage and this may account for the inferior flavour of imported meat when compared with home-killed.

The idea of such a disappearance was suggested by differences found when estimating carnosine in various samples of beef, by a colorimetric method previously described in this journal [Clifford, 1921]. Research on this extractive was begun just after the war when the beef commonly available was imported. Such samples gave yields of 0·35–0·37 % carnosine. On one occasion some English steak was obtained and gave the unexpectedly large yield of 1·1 %. It has been shown that for a given species the carnosine content of muscle is constant [Clifford, 1922] and the great difference between English and imported beef could not be explained unless cold storage had effected a reduction of the base. In order to test this various samples of English and imported meats were analysed.

Carnosine content of English and imported meat.

Beef.

Twelve separate samples of English beef have been examined over a period of two years with the following results: 1·1, 1·0, 1·1, 0·98, 0·96, 1·0, 0·97, 0·98, 1·1, 0·98, 0·98, 0·97 %. Average 1·0 %.

Five samples of imported beef gave: 0·37, 0·34, 0·36, 0·35, 0·36 %. Average 0·356 %.

Similar experiments have been carried out on veal, mutton and lamb and in every case the carnosine content of imported meat has been very much lower than that of English meat. Actual figures obtained were:

	English		Imported	
Beef	0·96–1·1 %	(12 samples)	0·34–0·37 %	(5 samples)
Veal	1·05–1·12	(5 ,,)	0·34–0·36	(4 ,,)
Mutton	0·37–0·38	(3 ,,)	0·13–0·16	(2 ,,)
Lamb	0·40–0·42	(2 ,,)	0·15–0·16	(2 ,,)

In all these experiments about one-third of the carnosine value was found in the imported meats as compared with English killed.

Experiments on the effect of cold storage on meat.

Since all the samples of imported meat were perfectly fresh and in an edible condition, the loss of carnosine could not be a putrefactive change, but might have been due to the length of time the meat had been in cold storage.

It was possible to test this idea owing to the kindness of Dr H. H. Dale who allowed a sample of English meat to be placed in a cold room in the Research Institute at Hampstead. Portions were removed at various times for analysis. When first put in, carnosine was present to the normal value of 0·99 %. Samples were taken at intervals of about one month. A fall of carnosine at first great, then slight, followed by a second steep fall was shown (Fig. 1).

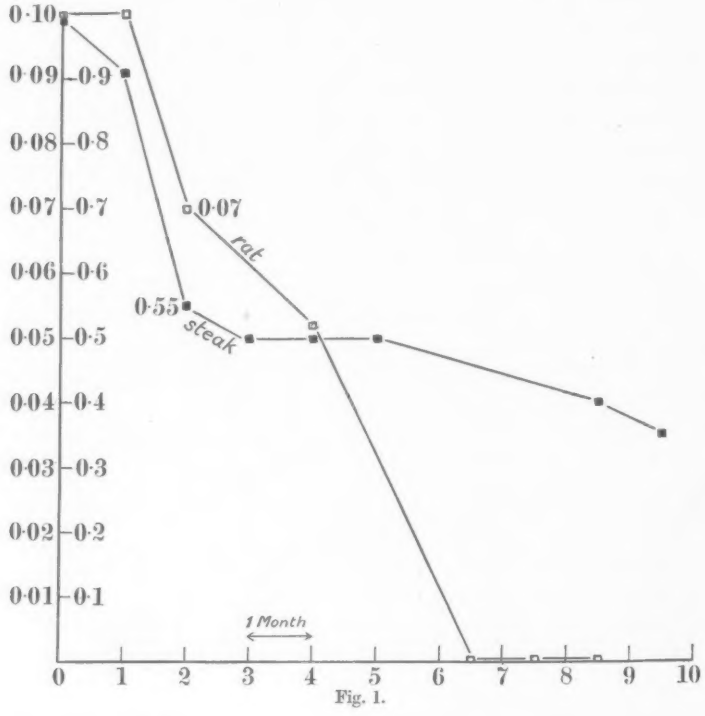


Fig. 1.

The actual figures were:

Original	0·99 % carnosine		4 months	0·50 % carnosine	
1 month	0·90	,,	6 ,,	0·48	,,
2 months	0·55	,,	8½ ,,	0·40	,,
3 ,,	0·50	,,	9½ ,,	0·35	,,

Effect of cold storage on rat muscle.

The loss of carnosine in beef might have originated in some process which took place before the meat was put into the cold room. Therefore seven rats were killed with coal gas and put into cold storage immediately after death. The normal value of 0·11 % carnosine was given by one animal, estimated directly, and the others were removed at intervals. The curve of carnosine loss ran parallel with that for beef for a period of four months (Fig. 1). This was followed by a steep fall in the fifth month and by six and a half months all trace of the base had disappeared.

Results were:

Original	0·11 % carnosine		$6\frac{1}{2}$ months	Absent
1 month	0·11	,,	$7\frac{1}{2}$,,	,,
2 months	0·07	,,	$8\frac{1}{2}$,,	,,
4 ,,	0·052	,,		

The above experiment shows that loss of carnosine occurs in muscle put into a cold room directly after the death of the animal in a similar manner to that shown by bought English meat kept at the same temperature.

The temperature of the cold room was just below 0° C. Spicules of ice were found in every sample analysed and the extractions were made before thawing out had completed.

Discussion of results.

From experimental results it may be stated that measurement of the depth of red colour produced on diazotising carnosine in a watery muscle extract gives an easy and rapid test for distinguishing between English fresh killed and cold storage meats. A sample of beef or veal with a carnosine percentage below 0·8 % or of mutton or lamb below 0·3 % would not come from a freshly killed animal, but probably from a carcase which had been chilled in order to preserve it. If the percentage were as low as 0·3 % in the case of beef or 0·15 % with mutton the sample would be 9–12 months old. Further experiments are in progress with the object of obtaining a curve by which the age of cold storage meat may be read off after determining the carnosine content.

The mechanism causing the loss of carnosine is unknown, but experiments are being carried out to investigate the problem. Since the reaction takes place at freezing point it is improbable that bacterial or enzyme action can account for the change, which is more likely to be initiated by a simpler type of catalyst.

Thanks are due to Dr H. H. Dale for allowing the meat to be deposited in the cold room at the Research Institute, Hampstead, and to Prof. V. H. Mottram for suggestions during the course of the work.

The expenses of this research were defrayed by the Medical Research Council.

REFERENCES.

Clifford (1921). *Biochem. J.* **15**, 400.
—— (1921). *Biochem. J.* **15**, 725.

XXIV. INVESTIGATIONS ON THE NITROGENOUS METABOLISM OF THE HIGHER PLANTS.

PART II.

THE DISTRIBUTION OF NITROGEN IN THE LEAVES OF THE RUNNER BEAN[1].

By ALBERT CHARLES CHIBNALL.

From the Biochemical Department, Imperial College of Science and Technology.

(*Received February 1st, 1922.*)

In the first part of this series [Chibnall and Schryver, 1921] a method of isolating part of the protein complex of fresh leaves was described. Subsequent work carried on with the same object, mentioned in that paper, in view, namely that of throwing light on the protein metabolism in the leaf, showed that it was necessary to investigate two problems in detail. Firstly the nature of the nitrogen retained in the residue of cellular matter after applying the above method remained to be determined, and secondly it was necessary to find out how far it is justifiable to compare leaves picked at different, and even unknown, periods of growth.

The present research on the leaves of the runner bean was primarily undertaken to solve these two problems. A method has been evolved whereby the whole of the nitrogenous material in the leaf is obtained in a state suitable for subsequent analysis. The water-soluble products are freed from all protein substances other than proteoses, about three-fourths of the protein N of the leaf is isolated as an amorphous powder, whilst the residue of the cell matter, containing 5–15 % of the total leaf N, is shown to contain only protein N. This makes it possible to estimate the total protein and water-soluble N in the leaf.

By applying this method at frequent intervals during the life history of the runner bean leaf, an attempt has been made to solve the second problem enumerated above. Not only has the ratio protein N to water-soluble N been found, but by suitable analysis either before or after hydrolysis, the distribution of the protein N in five groups, and of the water-soluble N in seven groups, has been determined.

A study of the protein groups indicates that the protein undergoes little, if any, change during the life history of the leaf, a fact that should be of assistance in future research into the chemistry of the leaf proteins. The water-soluble groups however are continually varying, indicating that comparisons of the composition of leaves, unless regard is paid to exact conditions

[1] Thesis approved for the Degree of Doctor of Philosophy in the University of London.

of age and growth, is almost impossible [compare Jodidi, Kellogg and True, 1920].

As the research progressed it was found possible to extend the scope of enquiry to the diurnal variations, and the effects of starvation, the results of which throw considerable light on the protein metabolism in the leaf. The synthesis of protein in the leaf from nitrates appears to take place through the amino acids, whilst the products of protein degradation, in striking analogy to what is found in the animal kingdom, pass into what appear to be urea derivatives.

A certain amount of research has been performed in the past to throw light on most of the problems discussed in this paper [see Czapek, 1920], but it was nearly all carried out before the development of modern methods for estimating the nitrogenous bases and monoamino acids, so that comparison of results is of very little value.

I. GENERAL EXPERIMENTAL METHODS.

Materials used. The plant used was *Phaseolus vulgaris* v. *multiflorus* (Scarlet Champion). The seeds were planted in unmanured ground following potatoes on the 28th April, 1921, at the Physic Gardens, Chelsea.

The seedlings appeared above the ground about 20 days later, and all ages of plants stated henceforth are calculated from the 18th May. The growth was normal, flowers appearing when the plant was five to six weeks old. At this time a few aphides appeared, but attention prevented them spreading, and all evidence of the disease had vanished three weeks later. By the middle of July it was found that although the inflorescence was normal, very few of the flowers had set, with the result that the number of pods formed was small, not averaging more than three or four per plant. This failure must be attributed to the abnormal dryness and high temperature of the atmosphere and not to the drought experienced during July, August and September, since the plants received abundance of water daily.

Methods of sampling. The procedure adopted was to pick the leaves from the plants selected, go lightly over the surface with a brush, remove all stems and take the total weight. It is from this weight, obtained before any appreciable evaporation from the surface of the leaves could have taken place, that the "N per fresh weight of the leaves," and hence all the figures given later in Tables XI and XIII, are calculated. The leaves were then counted, sorted out into six pans according to size, the content of each pan weighed, and the sample for grinding (200–250 g.) taken proportionately from each. By this means an average sample, giving a mean weight per leaf similar to that of the whole batch, was obtained. From several of the batches duplicates were taken to test the accuracy of the sampling and experimental methods used. The remainder of the batch was air-dried in an oven at 37° through which a slow stream of air was drawn.

Each batch picked was given a series number, which is retained throughout

in all subsequent operations. Details of the pickings, with the weather conditions, are given in Tables I and II. On account of the exceptionally fine weather conditions throughout the experiment the leaves were picked in a normal state without excess of moisture. Furthermore, the absence of high wind and storms kept the surface of the leaves clean and free from soil material. Only in one case, that of series 4, did rain fall in the preceding two or three days. In this rain fell during the night, but when the sample was taken in the morning no excess of moisture, as shown by lightly pressing between filter-papers, was found. Fresh weight determinations then can be considered as exceptionally good.

Dry weight and total nitrogen of the leaves. The air-dried remainder was used for this purpose. The dry weight was taken from a small sample placed for 24 hours in an oven heated to 108°.

The total N was determined by the modified Kjeldahl-Gunning method, whereby the nitrate N is first converted into amino-phenol by salicylic acid and sodium hyposulphite. The practical details followed were those given by Moore [1920].

General methods of manipulation (separation of protein, soluble products and cellular matter). Two methods which aim at this separation have so far been described, that of Osborne and Wakeman [1920] and the one communicated in the first paper on these investigations [Chibnall and Schryver, 1921]. In the former the leaves were passed through a Nixtamel mill three times; the pulp obtained mixed with water and returned twice more to the mill. The result was a green solution containing protein in colloidal suspension, from which the cellular matter was removed by centrifuging. The protein was afterwards flocculated by the addition of alcohol.

Briefly the second method consists of treating the minced leaves with ether-water and pressing out through thick muslin in a tincture press, whereby a green solution, containing most if not all of the water-soluble matter and part of the protein in colloidal suspension, was obtained. The protein was afterwards flocculated by warming. For the purpose of the present research this method, although easy to manipulate and rapid, was of little use unless the nitrogenous matter of the residue, about one-third of the total, could be separated from the cell matter for further investigation.

Osborne's method was certainly more thorough, the residue in this case containing only 18 % of the total N. It was inconvenient for the present purpose however, as it would have required the continuous use of a centrifuge. It was therefore decided to see if the ether-water method could not be improved.

Some cabbage leaves were accordingly treated by this method and the colloidal solution and residue examined microscopically, using a $\frac{1}{12}$th objective.

The solution appeared colourless, with two distinct sets of particles. The first, deep green in colour and irregular in shape, were obviously fragments of disintegrated chloroplasts, and were large enough to be free from movement. The second were very numerous, showed no trace of green colour, and

were small enough to be in rapid Brownian movement. On warming to coagulate the protein the particles were all removed, and the coagulate appeared as a greyish granular mass in which the fragments of the green chloroplasts could be distinctly seen.

The residue to the naked eye showed large disintegrated lumps of leaf matter. One of the smaller of these was examined under the microscope. The outer layer of cells had their walls broken and the major part of the content expelled; those inside were unruptured but flattened. In addition the vacuole content of the latter had been largely expressed, even though the layer of protoplasm was unbroken.

It seemed clear from this examination that the rupturing of some of the cells was due to the action of the cutter in the mincing machine, and that the function of the ether-water was merely to kill the semi-permeable membrane of protoplasm in the remainder, so that the fluid content could be squeezed out by the press. The large quantity of N remaining in the residue therefore was due to the protoplasmic membrane remaining in the unruptured cells. Clearly then to reduce the N content of the residue it was necessary to increase the number of cells ruptured.

No access could be had to a mill of the type used by Osborne. The only one available was that known technically as an End-runner Mill, which is nothing more than a mechanical pestle and mortar. It was found that if 200 g. of cabbage leaves were broken up and fed slowly into this, then 100 cc. of water added, the whole was reduced to a very fine green pulp in about 20 minutes. On pressing through muslin a solution, deeper coloured than before, was obtained. The grinding and pressing was repeated on the residue, and the N in the two colloidal extracts and final residue determined. The first extract contained 74·2 %, the second 14·8 %, and the residue 11 % of the total N.

Under the microscope the solution appeared much as before, except that the fragments of green chloroplasts were much more numerous and irregular in size, ranging from quite coarse lumps, which appeared however free from cell matter, down to small, but distinctly green, particles in Brownian movement.

The residue appeared different. The large masses of leafy matter had disappeared. Lumps of cell matter however could still be seen under the microscope, though the number of unruptured cells had greatly diminished. In addition numerous shreds of broken cells, containing only infrequent portions of protoplasm, were to be seen.

It is on this property—that the cellular matter is not ground down to a powder (but only ruptured and torn so as to set free the cell content)—that the success of the method, which was used throughout the present research, depends. The thick muslin and the tincture press were found to be quite unnecessary. All that is required is a glass funnel opening to a diameter of about 8 inches and a piece of fine cotton lawn 18 inches square. The whole

of the liquid, with its heavy charge of colloidal matter, is expressed by very slight hand pressure in under a minute, leaving behind a pasty mass that can be easily removed from the lawn and returned to the mill for further grinding. The fact that this fine lawn can be used renders the method applicable for quantitative work, since none of the cell matter or protein can be retained in its pores, and the amount of water-soluble N absorbed is negligible.

Reference to Table IV will show how complete a separation was obtained in the present research. Six grindings of the samples (200–250 g.) were given and the colloidal solution, as a safeguard against the pores of the lawn being enlarged by the passage of the liquid under slight pressure, was run through a second piece spread out over another funnel.

In several of the intermediate batches ether-water was used in place of distilled water, but no benefit was experienced and it was later discarded. In case some of the cells should have escaped disruption, and thereby still be retaining their water-soluble contents, the residue from the sixth grinding was thrown into 200 cc. of boiling water. The cellular matter at once swelled up, imbibing the water, and on cooling this was squeezed through the same lawn as before. The operation was then repeated. In the earlier workings, when the total N in the residue was low, very little N passed into these two extracts. In the later however, when the residual N rose to 12 % and over, sufficient to make an appreciable difference was found in the first, though a negligible quantity was always found in the second. The protein was flocculated in the first six series by warming to 45°, but the temperature was afterwards raised to 60°, since it was found that the precipitate was then rendered almost granular, and could be readily filtered in a few minutes, whereas before it had taken about an hour. The filtering was accomplished by means of a Soxhlet extractor thimble standing upright in a glass funnel. After all the liquid had drained through, the retained protein was washed with two extracts from the boiled residue described above. After draining a second time the thimble was then transferred to an extractor described by Schryver [1908] and washed for three or four hours with 95 % alcohol. As first filtered off the protein is a pasty mass, part of which clings to the sides of the thimble, but the majority collects in a large button at the bottom. As the alcohol washes it the surface becomes dehydrated and shrinks, thereby coming away from the wall of the thimble. The whole can then be shaken out and broken, so as to allow the alcohol access to a large surface and thus hasten the washing. It was returned to the thimble and after a further three or four hours in this extractor the thimble was removed and stood overnight in absolute alcohol. In the morning it was placed in a Soxhlet apparatus and extracted with ether until all colouring matter was removed. The protein was then shaken out of the thimble, ground up in a small mortar, returned to the thimble and left 24 hours to dry. The weight of the thimble was determined before the operation, and the increase when it contained the air-dried protein was taken as the weight of the latter.

In the previous communication by Chibnall and Schryver [1921] it was found that after the colloidal protein of cabbage was flocculated by warming, a secondary flocculation could be obtained by raising the temperature to about the boiling point. The same was found to occur in the present case, but the amount was small, and diminished when the temperature of the first flocculation was raised to 60°. Experiment on a moderate quantity of this second flocculent substance prepared from the cabbage showed that it was formed from some water-soluble protein by coagulation, since it was insoluble in all the solvents tried, and the percentage of N in it was about 12. In the present case it was separated by filtering on a Buchner funnel, and the filter paper, with the retained coagulate, transferred to a Kjeldahl flask and the total N estimated.

The aqueous extract, after boiling to remove all flocculent matter, was cooled, its volume taken, and stored in a stoppered bottle with a little chloroform and toluene for future use. As a precaution against any unknown reaction or separation on standing, the distribution of N in this was performed without delay. The alcohol and ether washings of the protein were evaporated down, transferred to a Kjeldahl flask and the total N estimated. The residue was air-dried at 37° in the same oven used for the main bulk of the leaves, as stated above. Total N was estimated by Kjeldahl's method.

It will not be out of place here to give some idea as to the time occupied in the separation. The grinding (10 mins. first, 6–7 mins. the others) and squeezing out take between 1 and $1\frac{1}{4}$ hours; raising the temperature to 60°, 5 mins., filtering through the Soxhlet thimble 15 mins., boiling the filtrate 10 mins., and the separation of the coagulated protein about 15 mins. About two hours, therefore, after the leaf cells are killed the colloidal protein is in alcohol, the clear aqueous extract has been boiled, cooled and shaken up with antiseptics, whilst the residue, containing none of the water-soluble N, is drying at 37°.

Distribution of N in the water-soluble products.

The total N in solution. Determined by the modified Kjeldahl-Gunning method mentioned above.

Nitric nitrogen. Reduction by Devada alloy in alkaline solution in place of the more usual Schulze-Tiemann method was employed, as the same apparatus as that required for free ammonia and amide N could be utilised. This consisted essentially of a two-necked Claisen flask without side tube, connected by means of an ordinary Kjeldahl steam trap to a double-surface condenser leading into the distillate flask of 500 cc. capacity containing $N/100$ sulphuric acid. The apparatus was set up in duplicate, the two larger flasks being immersed in the same water-bath. Both were also connected to the same vacuum pump (Geryk), so that similar conditions as to T and P were maintained in each.

The procedure was as follows: 50 cc. of the extract was run into each

flask late in the afternoon, then 10 cc. of 30 % NaOH, and into *one only* a small quantity of powdered Devada alloy. In this latter the evolution of hydrogen proceeded steadily during the night, with reduction of the nitric N to ammonia. Sufficient quantity of $N/100$ acid had been run into the receiver flasks to cover the tube leading from the condenser and the apparatus closed. In the morning 400 cc. of ammonia-free distilled water was run in by means of a tap funnel, the water-bath warmed to about 45° and the duplicate apparatus evacuated. Distillation was allowed to proceed for about two hours, 200 to 300 cc. of liquid coming over. The difference between the ammonia found in the two receivers was taken as that due to reduction of the nitric N. How necessary is the blank distillation with soda will be seen from the fact that the ammonia coming over was about twice that obtained from the same volume of extract using magnesia (as below), indicating a very slight decomposition by the strong alkali of some nitrogenous bodies (proteoses?) in the solution. Duplicate readings of nitric N by this method, without any regard being paid to the exact T or P, agreed to 0·2 cc., an accuracy in the solutions containing the lowest concentration of nitric N of 1 : 100, sufficient for the present research.

Ammonia nitrogen. 50 cc. or, if sufficient solution was available, 100 cc. of the extract were run into the Claisen flasks, then 400 cc. of ammonia-free distilled water followed by sufficient cream of magnesia to make the solution distinctly alkaline. Duplicate determinations were made at the same time. The amount of ammonia measured was equivalent to 1·5–3·5 cc. of $N/100$ acid, a quantity sufficiently small to be affected by errors due to the action of the MgO on the proteoses (see Nash and Benedict [1921] for discussion on methods of estimating free NH_3 content of blood). The figures given in the tables therefore only indicate the very low concentration of ammonium salts.

Amide nitrogen (*asparagine and glutamine N*). Sachsse's [1873] method was employed, whereby the extract was boiled with 4 % HCl for two hours. Sufficient extract to contain one-fourth to one-third of the total N in solution was used for this purpose. After this mild hydrolysis the solution was concentrated *in vacuo* almost to dryness to remove HCl, transferred with about 400 cc. of ammonia-free water to the Claisen flask described above, then cream of magnesia added to make the solution distinctly alkaline. The total ammonia present was found by distillation *in vacuo*, and from this the free ammonia originally present in the solution, as estimated above, was deducted. When the details of this research were planned, it was not realised that any quantity of proteoses would be present. It is possible that these might give amide N under the conditions stated above [see Osborne and Nolan, 1920], but the fact that no amide N at all can be detected in the samples picked at night shows that the error under this heading is not appreciable.

Humin nitrogen and nitrogen precipitated by phosphotungstic acid. After the distillation for amide nitrogen the procedure follows that of Hausmann for the hydrolysis products of protein, as modified by Osborne and Harris [1903].

Monoamino nitrogen. The filtrate and washings from the phosphotungstic acid precipitation were made just alkaline to litmus with 30 % NaOH, then just acid by a few drops of glacial acetic acid. They were then concentrated *in vacuo* to about 100 cc. and made up to a standard volume. Monoamino N was determined by Van Slyke's method.

"*Other nitrogen.*" Calculated by difference; the value is therefore subject to the sum of the individual errors in the six determinations mentioned above.

Proteose nitrogen. Estimated by saturation with zinc sulphate in acid solution. The precipitate obtained was filtered off, washed with a saturated solution of zinc sulphate until the washings were colourless, and redissolved in water. The solution was then again acidified, saturated with zinc sulphate, the precipitate washed as before, re-dissolved in water and made up to a standard volume. Total N was then estimated in an aliquot part by Kjeldahl's method.

Nitrous nitrogen. This was tested for by both the Griess and metaphenylenediamine reagents, but none was detected in any of the extracts (before or after heating) prepared in the present research.

Distribution of N in proteins.

Colloidal protein. Osborne and Harris' modification of Hausmann's method, referred to above, was used, monoamino N being determined by the method of Van Slyke.

Dried residue. When the total N in these (given later in Table IV) was determined they had not been washed in alcohol and ether to remove fats, lecithins, etc., since the extractors were being continuously used for work on the colloidal proteins. Series 7 B and 9 B, which were to be further investigated, were first washed with these solvents, when the N extracted was found to be respectively 0·60 % and 0·78 % of the total leaf N. These two extracted residues were then boiled up with 500 cc. of 1 % HCl for six hours under a reflux condenser, cooled, the undissolved portions filtered off, washed with water, and their N content determined by Kjeldahl's method. In terms of the total leaf N they contained 3·2 % and 4·13 % respectively. About three-fourths of the N left in the dried residue had therefore passed into solution. These extracts were then evaporated to a small bulk *in vacuo*, made up to 100 cc. so that they contained 20 % of HCl, hydrolysed for 16 hours, and the Hausmann numbers determined in a similar way to the colloidal protein above. They are given in Table VI later, and show that the N extracted was of protein origin. It is probable that the 3–4 % of N in the final residues mentioned above was also protein N that had escaped extraction by the dilute acid [compare Osborne and Wakeman, 1920].

Total protein and non-protein N. Previous work on the colloidal leaf proteins [Osborne and Wakeman, 1920; Osborne, Wakeman and Leavenworth, 1921; Chibnall and Schryver, 1921] indicates the possibility of a small

quantity of the N being of non-protein origin. In all the analyses therein mentioned, however, either hot or cold alkali was used for extraction, and since the resultant proteins have the properties of alkali-albumins, it is considered here that this "non-protein" N has been set free from the original protein complex by hydrolytic action. The constancy of the distribution of N in the colloidal proteins of the present research (Table V), especially in the starvation experiment to be described later, supports this view. In Table IV therefore the estimated total protein N is taken as the sum of the N in the colloidal precipitate, the dried residue and the protein coagulated by boiling.

There is evidence that more than one protein exists, amongst which may be cited the fact that the Hausmann numbers of the protein in the dried residues differ from those in the colloidal precipitate and also that a small part of the protein, as already mentioned, cannot be flocculated at 60°, but only precipitates when the liquid containing it is boiled. Furthermore, proteoses are present, but separate analysis to determine these could only be performed in the later series.

An analysis of the leaf N (series 9) in terms of protein N, proteose N and non-protein N is given in Table IX, whilst the distribution into seven groups after complete hydrolysis is given in Table X.

II. DESCRIPTION OF EXPERIMENTS.

Three main series of experiments have been carried out to determine:

(*a*) Seasonal variations.
(*b*) Diurnal variations.
(*c*) Results of starvation.

(*a*) *The seasonal variations.*

These were studied by a complete analysis of eleven batches of leaves picked at frequent intervals during the life of the plant. Except for the change of temperature at which the colloidal protein was flocculated from 45° to 60°, as stated earlier, the conditions of the individual analyses remained standard throughout.

(*b*) *Diurnal variations* (*series* 5–6 *and* 9–8).

These were studied at the end of the seventh and eleventh weeks. The day reading was obtained by picking the batch about half-an-hour before sunset, when the leaves had been subjected to 14 hours' light; the night reading by picking half-an-hour before sunrise, when the leaves had been in the dark about six hours.

From a climatic point of view the conditions under which these series were taken were ideal. In each case no rain had fallen for several days previously, nor was there any dew during the night, the relative humidity at the time of the night pickings being 85 % and 75 % respectively. This being so, the large decrease in the dry weight of the leaves during the night (10 % in both cases) can be taken as due entirely to translocation away from the leaf.

(*c*) *Starvation experiments* (*series* 10).

These were designed so to alter the conditions in the leaf that light would be thrown on the mechanism of either protein synthesis or degradation.

At the time that the batch for series 10 was picked two samples, 10 C and 10 D, were cut with long stems, placed in cups containing ordinary tap water so that their stems only were immersed, and left in a subdued light, 10 C for 100 hours and 10 D for 114 hours. In this way the leaves were kept quite fresh, but starved of nitrate and of sugar (other than that due to photosynthesis).

Table I. *Giving details of samples picked.*

Series number	Age above ground in days	Time of day when picked, G.M.T.	Date when picked	Condition of weather	Condition of plants
*1	14	8 A.M.	June 14	Sunny	Height 6–8 ins.
*2	21	,,	,, 21	Dull, no sun	Not very different from above
3	26	,,	,, 13	Sunny	Average height 12 ins. Just starting to climb
4	33	,,	,, 20	Dull, rainy night	Starting to flower
5	46	8.30 P.M.	July 3	Very fine day	Height 6–8 ft., flowers just developing
6	47	2.15 A.M.	,, 4	Fine night	As above
7	60	3 P.M.	,, 17	Very fine day	An appreciable increase of new shoots and leaves
†7N	66	2 A.M.	,, 23	Fine night	Pods just starting to form
8	77	,,	Aug. 3	,, ,,	Fully grown. Fertilisation had not taken place on account of the heat. A few pods still forming
9	81	3 P.M.	,, 7	Very fine day	Ditto
10	97	9 A.M.	,, 23	Fine day	Only a few pods fully formed. Numerous new shoots and leaves still forming
11	143	,,	Oct. 8	,, ,,	Most of the leaves old and beginning to show signs of chlorophyll degeneration. A few new shoots and leaves

* Planted 14 days later than the rest of the plants.

† No analyses were made with this batch.

Table II. *Giving further details of samples picked.*

Series number	Number of plants used	Average number of leaves per plant	Average weight of leaves per plant in g.	Average weight of leaf in g.
1	68	5·7	5·3	0·935
2	50	7·6	5·4	0·715
3	25	15·3	15·6	1·027
4	13	30·5	29·7	0·975
5 } day	12	56·3	50·9	0·904
6 } night	12	53·6	55·6	1·086
7	12	109·0	90·0	0·827
7N	12	—	126·0	—
8 } night	12	218·0	172·0	0·791
9 } day	12	259·0	202·0	0·781
10	6	372·0	245·0	0·683

Table III. (Compare Fig. 1.) *Showing the dry weight and total nitrogen.*

Series number	Fresh weight of sample in g.	Percentage dry weight	Percentage of N per fresh weight	Percentage of N per dry weight
1	160	11·43	0·623	5·44
2	88	13·06	0·671	5·13
3	177	13·43	0·658	4·90
4	191	13·16	0·588	4·48
5 day	193	15·85	0·678	4·28
6 night	236	14·46	0·653	4·51
7	590	15·90	0·762	4·76
8 night	1365	14·42	0·656	4·53
9 day	1925	15·51	0·675	4·35
10	921	16·31	0·724	4·44
11	164	16·13	0·622	3·86

Table IV. *Distribution of protein and water-soluble nitrogen in the leaves. (In percentages of total leaf N.)*

Series number	N of colloidal protein isolated	N of protein coagulated by boiling	N of residue	Estimated protein N *	N of alcohol and ether washings of colloidal protein	N remaining in solution after boiling
1	59·55	2·84	6·88	69·27	2·53	28·20
2	62·56	1·76	7·15	71·47	2·78	25·75
3	64·90	3·30	5·44	73·64	2·81	23·55
4	60·18	5·29	7·86	73·33	2·93	23·74
5A	56·32	3·61	13·34	73·27	2·44	24·29
5B	50·70	3·88	19·00	73·58	2·52	23·90
6A	56·02	2·97	13·37	72·36	2·75	24·89
6B	48·52	4·36	19·62	72·50	2·80	24·70
7A	59·61	1·34	12·25	73·20	2·46	24·34
7B	58·87	0·74	14·11	73·72	2·30	23·98
8A	61·28	0·68	14·92	76·88	2·02	21·10
8B	59·37	0·91	16·62	76·90	2·06	21·04
9A	58·11	0·68	16·81	75·60	2·72	21·68
9B	59·70	0·76	15·09	75·55	2·88	21·57
10A	56·82	0·68	18·17	75·67	2·17	22·16
10B	52·47	0·82	21·99	75·28	1·07	22·75
11	58·43	1·43	15·83	75·69	1·90	22·41
Starvation experiments:						
10C	49·55	1·79	12·30	63·64	2·22	34·14
10D	51·85	0·72	8·75	61·32	2·50	36·18

* The sum of the three columns on the immediate left.

Table V. *Percentage distribution of nitrogen in the colloidal protein.*

Series number	Amide N	Humin N	Basic N	Monoamino N	"Other" N
1	7·44	3·52	21·65	58·90	8·49
2	6·56	3·50	22·40	60·25	7·29
3	6·68	3·30	21·56	58·60	9·86
4	6·59	3·63	21·08	58·20	10·50
5A day	6·68	4·18	20·55	59·67	8·78
6B night	6·36	3·64	19·82	59·54	10·64
7B	6·37	3·57	20·55	59·10	10·41
8B night	6·14	3·55	19·33	59·25	11·73
9B day	6·34	3·64	22·00	58·88	9·14
10B	6·50	3·82	21·78	59·22	8·68
*10C	6·60	3·56	21·63	58·50	9·71
11	6·40	3·82	21·32	58·50	9·96

* Starvation experiment.

Table VI. *Showing the distribution of N in the products extracted from the residues by 1 % HCl (after complete hydrolysis).*

Series number	Amide N	Humin N	Basic N	Monoamino N	"Other" N
7B	8·38	5·73	25·18	48·20	12·53
9B	8·19	5·82	24·66	48·78	12·64

Table VII. *Distribution of the water-soluble nitrogen (in percentages of total leaf nitrogen). After hydrolysis with 4 % HCl for two hours.*

Series number		Ammonia N	Asparagine N	Nitric N	Humin N	Proteose + basic N	Mono-amino N	"Other" N
1		0·67	0·79	7·78	0·78	7·63	6·43	4·64
2		0·22	0·62	7·35	0·79	6·77	6·41	3·59
3		0·27	0·94	3·93	0·72	6·29	5·54	5·87
4		0·27	0·51	2·45	0·74	6·67	5·52	7·58
5A	day	0·47	0·49	2·71	1·32	6·81	9·57	2·92
5B	day	0·36	0·43	2·96	0·66	6·25	9·25	3·99
6A	night	0·45	nil	3·89	1·19	6·60	10·37	2·39
6B	night	0·34	nil	3·66	1·07	6·79	10·16	2·77
7A		0·39	0·21	3·77	0·83	7·90	9·33	1·91
7B		0·32	0·24	4·25	1·06	7·49	8·60	2·02
8A	night	0·34	trace	3·38	0·94	6·87	7·08	2·49
8B	night	0·38	trace	3·74	1·07	6·62	7·05	2·18
9A	day	0·41	0·48	3·04	1·03	6·67	7·72	2·33
9B	day	0·44	0·53	2·75	0·99	6·35	7·99	2·45
10B		0·37	0·47	3·53	1·06	6·89	6·81	3·62
11		0·61	0·92	1·28	0·96	6·97	6·61	5·05

Table VIII. *Showing the percentage of nitrogen found in the colloidal precipitates.*

Series number	Percentage of nitrogen	Series number	Percentage of nitrogen	Series number	Percentage of nitrogen
1	11·42	6A	11·84	9A	11·66
2	11·44	6B	10·75	9B	11·71
3	11·76	7A	11·75	10A	11·54
4	9·73	7B	11·81	10B	11·36
5A	10·88	8A	11·44	10C *	11·35
5B	10·68	8B	11·75	10D	11·42

* Starvation experiment.

Table IX. *Showing the amounts of protein, proteose and non-protein nitrogen in the leaves of series 9 (age 14 weeks).*

	Percentage of total N
Colloidal protein	59·70
Protein in residue extracted by 1 % HCl	10·24
Undetermined in residue	4·13
Coagulated by boiling	0·76
Total protein N	74·83
Proteose N	4·35
Non-protein N, soluble in water... ...	17·22
Total	96·40
Soluble in alcohol and ether	3·60

Table X. *Showing the final analysis of the leaf nitrogen in series* 9 B *(in terms of total leaf N). After complete hydrolysis.*

	Ammonia N	Nitric N	Amide N	Humin N	Basic N	Mono-amino N	"Other" N
Colloidal protein	—	—	3·78	2·17	13·13	35·16	5·46
Residue	—	—	1·18	0·82	3·54	7·01	1·82
Proteoses, water-soluble N	0·44*	2·73*	1·26	1·08	2·78	9·01	4·25
Coagulate	—	—	—	—	—	Total N	0·76
Soluble in alcohol and ether	—	—	—	—	—		3·60
Total	0·44	2·73	6·22	4·07	19·45	51·18	15·89

* Determined before hydrolysis.

Table XI. (Compare Fig. 1.) *Showing the amounts of protein N, water-soluble N and the components of the water-soluble N in percentages of the fresh weight of the leaves.*

Series number		Protein N	Water-soluble N	Ammonia N	Nitric N	Asparagine N	Humin N	Proteose + basic N	Mono-amino N	"Other" N	
1		0·4315	0·1757	0·0042	0·0485	0·0049	0·0048	0·0475	0·0401	0·0289	
2		0·4795	0·1728	0·0045	0·0493	0·0041	0·0053	0·0454	0·0430	0·1241	
3		0·4846	0·1550	0·0017	0·0259	0·0062	0·0047	0·0414	0·0358	0·0386	
4		0·4322	0·1396	0·0016	0·0144	0·0030	0·0043	0·0392	0·0325	0·0446	
5A	day	0·4967	0·1647	0·0027	0·0192	0·0031	0·0067	0·0444	0·0637	0·0237	The last seven columns are the mean of the respective A and B values
5B	day	0·4989	0·1620								
6A	night	0·4725	0·1626	0·0026	0·0247	0·0	0·0072	0·0438	0·0670	0·0168	
6B	night	0·4732	0·1613								
7A	day	0·5575	0·1855	0·0026	0·0291	0·0016	0·0072	0·0567	0·0683	0·0149	
7B	day	0·5620	0·1828								
8A	night	0·5045	0·1382	0·0024	0·0234	0·0	0·0066	0·0443	0·0463	0·0152	
8B	night	0·5045	0·1380								
9A	day	0·5102	0·1463	0·0028	0·0196	0·0034	0·0067	0·0439	0·0523	0·0172	
9B	day	0·5100	0·1458								
10A		0·5477	0·1605	—	—	—	—	—	—	—	
10B		0·5450	0·1647	0·0027	0·0256	0·0034	0·0077	0·0499	0·0493	0·0262	
11		0·4708	0·1394	0·0038	0·0080	0·0058	0·0059	0·0423	0·0411	0·0314	

Table XII. *Showing variations introduced in the distribution of the water-soluble N of series* 10 *by starvation.*

(Figures given are in percentages of the total leaf N)

Series number	Hydrolysis: Strength of HCl	Hydrolysis: Time in hours	Nitric N	Ammonia N	Asparagine N	Amide N other than asparagine N	Humin N	Proteose N	Basic N	Mono-amino N	"Other" N
10A & B	4%	2	3·53	0·37	0·47	—	1·06	4·59	2·30	6·81	3·62
10C (1)	4	2	3·42	0·46	0·49	—	0·88	(3·60)[1]	0·67	6·95	17·66
10C (2)	20	16	3·42	0·46	0·49	5·69	1·71	—	5·03	8·88	8·46
10D (1)	—	—	—	—	—	—	—	3·52	—	—	—
10D (2)	20	16	(3·42)[2]	(0·46)[2]	(0·49)[2]	6·57	1·72	—	5·61	9·64	8·27
9B (1)[3]	4	2	2·73	0·44	0·53	—	0·99	4·35	2·00	7·76	2·77
9B (2)[3]	20	16	2·73	0·44	0·53	0·73	1·08	—	2·78	9·01	4·25

[1] Estimated by comparison with 10D (1).

[2] Not determined; assumed, by comparison with 10C, to be unchanged.

[3] Extract 9B was used as none of 10A or 10B was available.

Table XIII. *Showing the diurnal variation in the N of series* 5–6 *and* 9–8, *in percentages of the fresh weight of the leaves.*

Series number		Total N	Protein N	Water-soluble N	Ammonia N	Nitric N	Asparagine N	Humin N	Proteose + basic N	Monoamino N	"Other" N
5A, 5B	day. Mean	0·6780	0·4980	0·1634	0·0027	0·0192	0·0031	0·0068	0·0444	0·0637	0·0237
6A, 6B	night. Mean	0·6530	0·4730	0·1620	0·0026	0·0247	0·0	0·0072	0·0438	0·0670	0·0168
Diurnal difference		−0·0250	−0·0250	−0·0014	−0·0001	+0·0055	−0·0031	+0·0004	−0·0006	+0·0033	−0·0069
9A, 9B	day. Mean	0·6750	0·5101	0·1460	0·0028	0·0196	0·0034	0·0067	0·0439	0·0523	0·0172
8A, 8B	night. Mean	0·6560	0·5045	0·1392	0·0024	0·0234	0·0	0·0066	0·0443	0·0463	0·0152
Diurnal difference		−0·0190	−0·0056	−0·0068	−0·0004	+0·0038	−0·0034	−0·0001	+0·0004	−0·0060	−0·0020

\+ Indicates that there is a diurnal increase. − Indicates that there is a diurnal decrease.

Table XIV. *Showing the difference between certain duplicate readings in Tables IV and VII.* (*Expressed as a percentage of the mean value of the two readings.*)

Series number	Protein N	Water-soluble N	Nitric N	Monoamino N	"Other" N
5A, 5B	0·44	1·69	8·44	3·37	25·0
6A, 6B	0·15	0·83	5·91	2·05	14·0
7A, 7B	0·75	1·48	11·30	8·49	5·5
8A, 8B	0·0	0·15	9·60	0·43	12·0
9A, 9B	0·0	0·50	9·54	3·50	6·0
10A, 10B	0·50	0·77	—	—	—

The results of the above experiments are given in Tables III to XI. All the figures in the hydrolyses are the means of duplicates. Since the fresh weight of the sample taken for grinding might be too low on account of evaporation from the surface of the leaves whilst sorting, the distribution of N was calculated in terms of the total N. To convert this distribution into one in terms of the fresh weight of the leaves, as is given in Tables XI and XIII, the appropriate value for "N per fresh weight" given in Table III was used. In connection with these tables the following definitions must be noted:

(1) "*Amide N*" in Tables V and VI. This is used in the standard way to denote the ammonia set free on the complete hydrolysis of proteins, etc.

(2) "*Asparagine N.*" Ammonia obtained by hydrolysis with 4 % HCl for two hours (after deduction of free ammonia nitrogen). (See reservation *re* Osborne and Nolan given on p. 350.)

(3) "*Amide N other than asparagine N.*" In Table XII this is the ammonia set free on complete hydrolysis minus that estimated as asparagine in (2), and as free ammonia.

(4) "*Monoamino N*" refers only to the amino N that is estimated by Van Slyke's method.

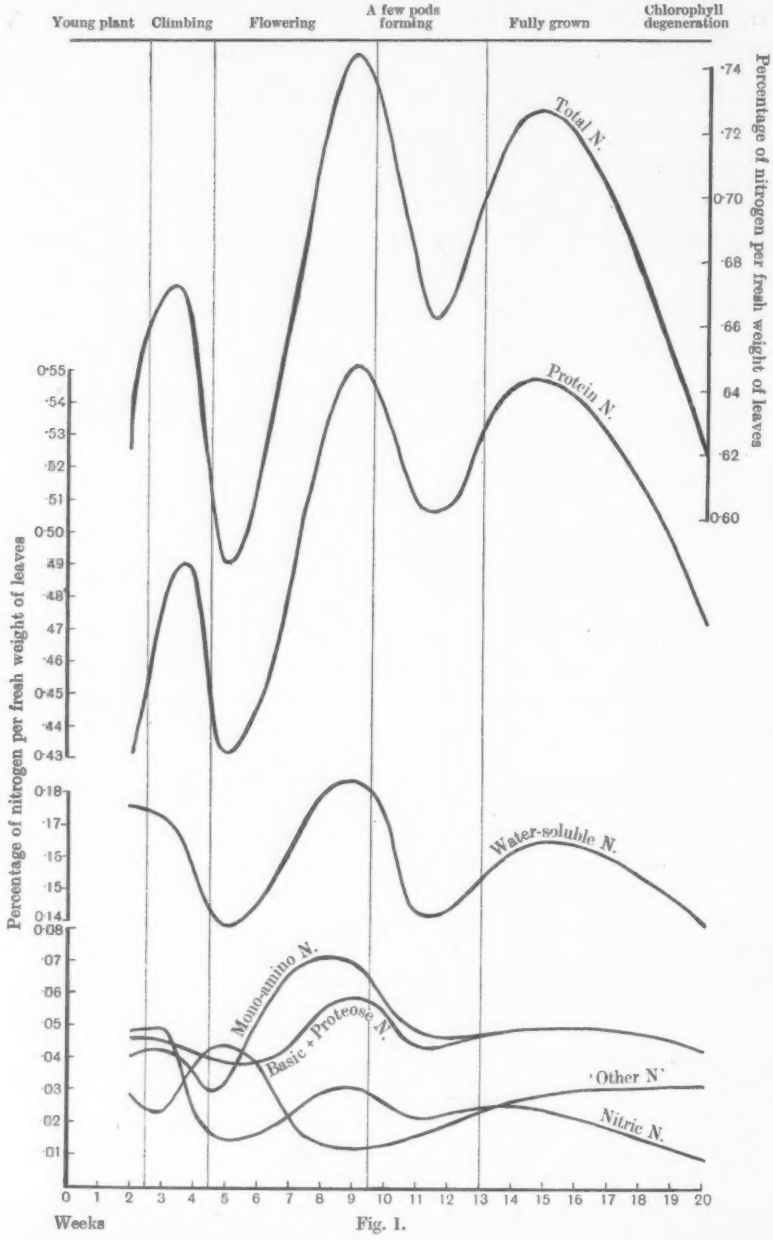

Young plant
Climbing
Flowering
A few pods forming
Fully grown
Chlorophyll degeneration
Total N.
Protein N.
Water-soluble N.
Mono-amino N.
Basic + Proteose N.
'Other N'
Nitric N.
Percentage of nitrogen per fresh weight of leaves
Percentage of nitrogen per fresh weight of leaves
Weeks

Fig. 1.

(5) "*Other N*" is the N not estimated in any of the other groups given. Calculated by difference.

(6) "*Protein N*" is the sum of the N in the colloidal precipitate, dried residue and coagulated protein found by boiling the extract (see p. 351).

Fig. 1 illustrates Table XI. The curves are drawn to show a value for about 9 a.m. to 11 a.m. (after 6–8 hours daylight). Whenever only sunrise and sundown values have been determined, the value shown on the curves (Fig. 1) is the mean of the two.

Sampling and experimental errors in the distribution of N[1]. In no case was it possible to take more than duplicate samples of the same batch of leaves, so that probable errors, based on Peter's formula, cannot be obtained. Table XIV however shows the differences between duplicate samples of the more important groups given in Tables IV and VII calculated as a percentage of the mean reading. The protein N shows a maximum difference of 0·75 % only, so that all the changes indicated in Fig. 1 can be taken as significant. The water-soluble N shows a maximum difference of 1·69 %. The nitric N shows a maximum difference of 11·3 % and a minimum of 5·91 %. This is much greater than the experimental error, and must be due to variation of the concentration in different leaves. As none of the changes indicated by Fig. 1 is less than 20 % they can be regarded as significant.

Monoamino N shows a maximum difference of 8·49 %, although that in the other four readings does not exceed 3·5 %. The experimental error here is undoubtedly high, due to the small volume of N actually measured, but a variation of 12 % or over should be fairly significant.

"Other N" is calculated by difference, and shows a maximum of 25 %. Considering that it is subject to all the errors in the rest of the N groups, a variation of 50 % is required to be at all significant.

III. DISCUSSION OF RESULTS.

The main results, as indicated in the tables and figure, may be summarised as follows:

(*a*) *Seasonal variations.* (Tables I, II, III, V; Fig. 1.)

(1) Total N and protein N, calculated as percentages of fresh weight, vary with growth, decreasing when this is very rapid or pods are forming.

(2) The distribution of N in the colloidal protein remains practically unchanged throughout the series.

(3) Asparagine N and free ammonia N remain low throughout the series.

(4) Nitric N and monoamino N vary directly with the protein N, indicating that they may be connected with protein synthesis. Similarly the "other N" varies inversely as the protein N, indicating that it may be connected with protein degradation.

[1] Sampling errors for dry weight and total N were not obtained in the present research. Earlier work on the leaves of the broad bean showed, for a batch of 250 g., the following probable errors calculated by Peter's formula: dry weight, 0·48 %; N per dry weight, 0·63 %; N per fresh weight, 0·91 %.

(5) The water-soluble products show considerable variation throughout the series, indicating that comparison of leaves from plants of different age is impossible [see Jodidi and colleagues, 1920].

(*b*) *Diurnal variations.* (Table XIII.)

(1) There is a diminution of total solids at night.

(2) This is accompanied by a diminution of total N and protein N.

(3) There is a distinct rise in the nitric N at night.

(4) The asparagine N disappears at night.

(5) The other water-soluble products remain more or less unchanged in amount during the night.

(6) The most significant diminution of nitrogenous products at night must be assigned to the proteins, indicating translocation of unchanged protein or its decomposition products.

The results for series 9–8 are not so reliable, since three days elapsed between the pickings. The conclusions to be drawn from them are similar to those stated above, except that there is a loss of both water-soluble N and monoamino N. This difference may be connected with the fact that pods were forming at the time.

(*c*) *Starvation experiments* (series 10). Tables IV, V and XII.

(1) There is a considerable decrease of protein N, with increase of water-soluble N.

(2) The distribution of N in the colloidal protein remains unaltered.

(3) Nitric N and ammonia N remain unaltered.

After mild hydrolysis:

(4) Asparagine N and monoamino N remain unchanged.

(5) Proteose N and basic N have decreased.

(6) There is a large increase of "other N," equivalent to the decrease in the protein N.

After complete hydrolysis:

(7) The "other N" is greatly reduced, and allowing for proteoses in solution, half of the loss reappears as ammonia (amide N other than asparagine N in Table XII) and one-third as bases.

Perhaps the most interesting of these results is that the protein disappears, and is replaced, not as one might expect by amide N and amino N, which remain more or less constant, but by N products of undetermined composition, given under the heading "other N." Light is thrown on the nature of these products by the different results of mild and complete hydrolysis. It would appear that they consist, in large part, of substances that fulfil the following conditions:

After mild hydrolysis:

(1) Yield no free ammonia, *i.e.* contain no acid-amide linkage R—CO—NH_2.

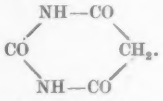
(2) Give no precipitate with phosphotungstic acid.

(3) Give no increase of free amino groups capable of reacting with nitrous acid.

After complete hydrolysis:

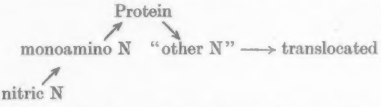
(4) Break down giving about one-half their N as ammonia and one-third as bases.

This great increase of free ammonia on complete hydrolysis suggests a urea derivative in which the amino groups are coupled to form compounds capable of resisting mild hydrolysis. The ureides are substances of this type; further they are, as a class, strongly acidic and capable of forming salts. As an example take barbituric acid,



This substance contains both replaceable imino and methylene hydrogen atoms, and only on prolonged hydrolysis breaks down giving ammonia from the urea nucleus. A condensation product of such an acid with one or more of the nitrogenous bases might possibly resist disruption on mild hydrolysis, and so give no precipitate with phosphotungstic acid due to liberated bases. In the absence of further chemical evidence this view can only be regarded as a speculation, but it is worth noting that these products appear to be present in small quantities in the unstarved leaf extract. A complete hydrolysis (see 9 B (1) and (2), Table XII) of this extract gives an increase of ammonia equivalent to 17 % of the proteose N, a value three times greater than one would expect from published data concerning the amide N content of proteoses. Furthermore, Fosse [1912, 1913, 1914] has demonstrated the presence of traces of urea in the leaves of higher plants.

Summarising the results under (*a*), (*b*) and (*c*) above, it would appear that the nitrogenous metabolism of the leaf runs on the following lines:



This will explain the following points brought out in the above discussions:

(1) The graphs of the nitric and monoamino N in Fig. 1 vary with that of the protein N.

(2) In spite of rapid protein degradation at night, there is very little change in most of the constituents of the water-soluble N.

(3) There is an accumulation of "other N" during the fourth and fifth weeks when protein degradation appears to be rapid, also later in the season when the plant is becoming aged, and translocation less active.

(4) In the starvation experiment, though protein degradation is rapid, there is no increase in amide N or monoamino N, and, since translocation away from the leaf has stopped, there is a large increase in "other N."

Protein metabolism, however, does not depend on N alone, and due regard must be paid to the supply of carbohydrate and other substances when interpreting some of the results outlined above. Thus in the starvation experiments,

since nitric N is present, it might be asserted that protein synthesis would go on until this was used up. But in this case the leaves were starved of sugar, and since they were placed in a subdued light photosynthesis would be weak. In all probability then, protein synthesis had stopped for lack of carbohydrate [compare Suzuki, 1898]. Similarly the accumulation of nitric N at night is probably due to the fact that starch, stored during the day, has been partially or entirely used up, so that the supply of carbohydrate for protein synthesis is reduced or is stopped.

The low concentration of free ammonia and asparagine N observed throughout the series confirms the work of Emmerling [1887] on the broad bean, and of Kosutany [1897] on the vine. The researches of Wasilieff [1901] and Prianischnikoff [1899] have shown that the growth of seedlings depends in part on asparagine derived from the decomposition of the reserve proteins in the cotyledons. This may indicate that the function of asparagine in the general metabolism of the plant is connected with growth, so that the total disappearance at night, an observation confirmed by Kosutany, may be due to translocation away from the leaf to the growing points or roots. The starvation experiments, where the asparagine N remains unchanged, support this view, and show that this substance is not derived from the decomposition of the leaf proteins. No indication as to its origin can be deduced from the results of the present research, but they lend support to the theory of Butkewitsch [1909] that its formation is to remove excess of free ammonia.

This research was carried out at the instigation of Prof. S. B. Schryver, whom the author thanks, together with Prof. V. H. Blackman, F.R.S., for continued advice and criticism. His thanks are also due to Mr Hales, of the Chelsea Physic Gardens, for the care bestowed on the plants used.

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REFERENCES.

Butkewitsch (1909). *Biochem. Zeitsch.* **16**, 411.
Chibnall and Schryver (1921). *Biochem. J.* **15**, 60.
Czapek (1920). Biochemie der Pflanzen (Jena), II, pp. 291–307.
Emmerling (1887). *Land. Vers. Stat.* **34**, 1.
Fosse (1912). *Compt. Rend.* 155, 851.
—— (1913). *Compt. Rend.* 156, 1934.
—— (1914). *Compt. Rend.* 158, 1374; 159, 253.
Jodidi, Kellogg and True (1920). *J. Amer. Chem. Soc.* **32**, 1061.
Kosutany (1897). *Land. Vers. Stat.* **48**, 13.
Moore (1920). *J. Ind. Eng. Chem.* **12**, 669.
Nash and Benedict (1921). *J. Biol. Chem.* **48**, 2.
Osborne and Harris (1903). *J. Amer. Chem. Soc.* **25**, 323.
—— and Nolan (1920). *J. Biol. Chem.* **43**, 2.
—— and Wakeman (1920). *J. Biol. Chem.* **42**, 1.
—— Wakeman and Leavenworth (1921). *J. Biol. Chem.* **49**, 1.
Prianischnikoff (1899). *Land. Vers. Stat.* **52**, 137, 347.
Sachsse (1873). *J. pr. Chem.* n. F. **6**, 118.
Schryver (1908). *J. Physiol.* **37**. *Proc.* xxiii.
Suzuki (1898). *Bull. Coll. Agr. Tokyo*, **2**, 409.
Wassilieff (1901). *Land. Vers. Stat.* **55**, 45.

XXV. MILK AS A SOURCE OF WATER-SOLUBLE VITAMIN. III[1].

By THOMAS BURR OSBORNE and LAFAYETTE BENEDICT MENDEL

WITH THE COOPERATION OF

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(*Received February 14th, 1922.*)

The question of the vitamin content of milk has, very properly, attained considerable prominence not only in its scientific aspects in relation to nutrition but also from the standpoint of public health. The classic experiments of F. G. Hopkins [1912] indicating an unexpectedly favourable effect upon health and growth secured by the addition of as little as 2 cc. of cow's milk per day to a "synthetic diet" fed to young rats have given the occasion for the current emphasis upon the richness of milk in that nutritive factor now commonly designated as vitamin *B*. Our own early experiments [1911] published in 1911 had indicated that what we originally termed "protein-free milk"[2] furnishes something without which rats cannot grow satisfactorily when they are kept upon "synthetic" diets consisting of mixtures of more or less isolated food substances. However, the quantities of "protein-free milk" which we have found necessary to supply the essential food factor to rats in numerous trials at various times have been decidedly larger than would correspond to 2 cc. of milk. This might have been due to a variety of obvious possibilities including the loss of vitamin in the process of preparation of the milk product. Consequently we [1918] undertook a re-investigation of the subject, using milk as the added source of vitamin *B* in the ration. The experiments indicated the necessity of feeding as much as 16 cc. of fresh cow's milk to secure the desired growth. Again [1920] we made further attempts to demonstrate a possible greater vitamin *B* potency by feeding unpasteurised milk of known origin. In some of these trials the diets used by Hopkins were imitated as closely as the materials at our disposal would permit. Again "the outcome

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, Washington, D.C.

[2] This product consists of the dried solids of skim milk from which the casein has been removed by precipitation with acid and the coagulable proteins removed by heat.

was in harmony with all our experience in showing that even additions of 10 cc. of fresh milk per day were insufficient to effect a food intake adequate for growth at a normal rate" [1920].

Neither seasonal variations, differences in the rations fed to the lactating cows, nor manipulations of the milk prior to marketing appeared to offer a satisfactory explanation of the differences between the results recorded by Hopkins and ourselves. He [1920][1] has lately reported the outcome of a new series of tests of 2 cc. of milk per day as a source of vitamin *B* for rats. His experiences are related as follows:

"On receipt of Osborne and Mendel's private communication, I made a few experiments in the winter of 1919. At this time the results were frankly disappointing. When the animals receiving the 2 cc. of milk in addition to the synthetic diet were compared with others which had the latter alone, the favourable effect upon health and upon the survival periods of the animals was unmistakable. Growth, however, was very slow, and the death rate of the rats was higher than that of animals normally fed.

"In April of this year fresh experiments were begun and continued during the summer. The results now became such as to confirm entirely my earlier experiments. Out of 20 animals, each receiving daily 2 cc. of fresh cows' milk as an addition to a highly purified synthetic diet, not one failed to grow almost normally throughout the period of experiment. The observations were not extended beyond 60 days, as they were meant only for comparison with those described in my original paper, and not to determine the amount of milk necessary for continued growth. The technique was exactly that originally used by me. In each experiment control animals were fed upon the synthetic diet alone and the contrast between them and a corresponding set receiving 2 cc. of milk was just as marked as in my original experiments. There has been no selection of results."

It will be noted in most of Hopkins' experiments that he began the feedings when the rats were still quite young, often weighing not more than 40 g. We concluded to repeat our tests upon rats somewhat younger than those with which our previous experiments were begun. The standard diet consisted of

Casein	18 %	Butter fat ...	9 %
Salt mixture[2] ...	4	Lard	20
Starch	54		

Each day 2 cc. of milk, supplied in a separate dish, were promptly consumed by the rats. The tests began October 19, 1921. The milk was obtained unpasteurised from a nearby supply. A bacterial count made in November on samples from the same source showed 230,000 bacteria per cc.

[1] In this paper a reference (p. 724) to previous experiments by Osborne and Mendel is erroneously cited as *Biochem. J.* **41**, 515. It should be *J. Biol. Chem.* **41**, 515.

[2] [Osborne and Mendel, 1919.]

The changes in body weight are shown graphically in the appended chart, the weekly food intakes (exclusive of milk solids) being indicated, where available, on the curve of growth. During the periods represented by the broken lines (- - - - - -) 40 mg. per day of a yeast fraction prepared by Osborne and Wakeman's method [1919] replaced the milk in the case of rats 7462, 7485, 7488, 7489. Rat 7506 received 0·1 g. dried brewery yeast per day. Rat 7460 died with symptoms characteristic of lack of vitamin *B*.

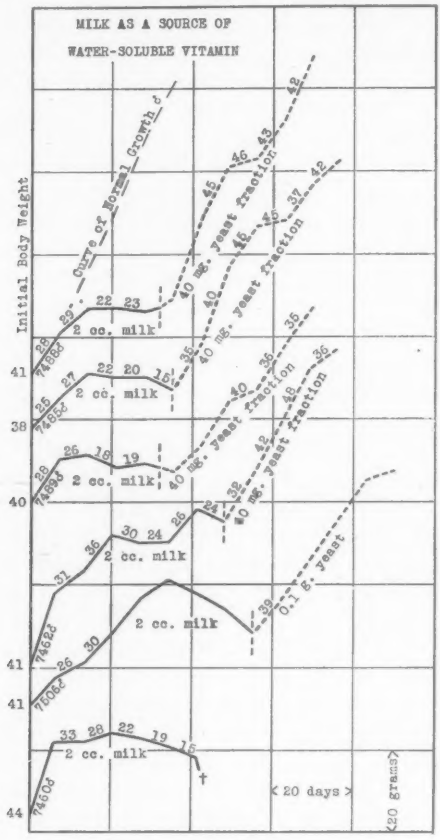

Fig. 1.

If variations in the vitamin *B* content of cow's milk from different sources actually reach the magnitude represented by the discrepancies between the English reports and our own they can scarcely be due to variations in the diet of the cows. The differences seem too large to be accounted for by such a probability. Hopkins himself states:

"The incomplete observation just mentioned, and the experience of Osborne and Mendel, suggest, at any rate, that the apparent seasonal variation in the results of my experiments was not likely to be due to differences in the milk. That there is a seasonal factor in the growth energy of rats is I think sure, but it is doubtful if it could account for the large difference in the experimental results now described. I am endeavouring to obtain further light on the matter."

In harmony with our own experiences alike with fresh cow's milk and "protein-free milk" prepared therefrom are reports from other investigators. Thus Johnson [1921], who studied the "growth-promoting properties" of milk and dried milk preparations used in the form of "reconstructed" milk in the Hygienic Laboratory of the U.S. Public Health Service, found that his animals required, as an adequate source of vitamin *B*, an amount of the milk equivalent to that reported by ourselves. Thus he states that "a food mixture consisting of purified foodstuffs plus milk as the sole source of water-soluble vitamine must contain at least 2½ parts of milk to 1 part of the basal ration in order to produce normal growth. Since a full-grown male rat weighs about 280–300 grams, and a full-grown female about 180 grams, a rat upon such a diet consumes just about 16–18 cubic centimeters of milk daily. These figures agree with those of Osborne and Mendel. It is also seen that the rate of growth of rats receiving less than 2½ parts of milk in their diet was accelerated after increasing the amount of milk in the diet."

According to Johnson and Hooper [1921] "Pigeons fed upon mixtures of spray process skim milk powder with polished rice require 30 per cent. of the food in skim milk powder in order to get full protection from polyneuritis. This corresponds to about 75 cc. daily liquid milk."

Dutcher [1921], Dutcher, Kennedy and Eckles [1920], Dutcher and colleagues [1920, 2], have found spring milk obtained after the cows were placed on green grass to be superior to winter milk in "antiscorbutic and nutritive properties." However, when the milk was at its best it required 10 cc. to furnish "sufficient water-soluble and fat-soluble vitamine for normal growth in the albino rat." Even the greatest reported increases in antiscorbutic potency in the milk of cows fed upon especially suitable rations [Hart, Steenbock and Ellis 1920; Dutcher and colleagues, 1920, 1; Hess, Unger and Supplee, 1920] do not parallel the increment which the differences represented by 2 cc. (Hopkins) and 10 cc. respectively per day denote. If the variations in the vitamin-content of the diet of cattle in different places will account for the marked differences in the vitamin *B* content of cow's milk they must be far more pronounced than has been suspected hitherto.

Through the cooperation of the Department of Obstetrics in the School of Medicine, Yale University, we have been enabled to test mixed samples of human milk for its content of vitamin *B* in the same way as has been reported for cow's milk. The product, which represented the secretion from several persons, was not essentially richer than we have found cow's milk

to be. 5 cc. of human milk per day added to the standard diet devoid of vitamin *B* was insufficient to secure continued increments of body weight. When the milk was desiccated and fed in the form of tablets the equivalent of 10 cc. sufficed, in addition to the standard food eaten, to secure growth at a fairly good rate. In no case was the human milk equivalent in its potency as a source of vitamin *B* to the cow's milk used in Hopkins' most successful tests.

REFERENCES.

Dutcher (1921). *J. Ind. and Eng. Chem.* **13**, 1102.
Dutcher, Eckles, Dahle, Mead and Schaefer (1920, 1). *J. Biol. Chem.* **45**, 119.
Dutcher, Eckles, Dahle, Mead and Schaefer (1920, 2). *Science*, **52**, 589.
Dutcher, Kennedy and Eckles (1920). *Science*, **52**, 588.
Hart, Steenbock and Ellis (1920). *J. Biol. Chem.* **42**, 383.
Hess, Unger and Supplee (1920). *J. Biol. Chem.* **45**, 229.
Hopkins (1912). *J. Physiol.* **44**, 425.
—— (1920). *Biochem. J.* **14**, 721.
Johnson (1921). *Public Health Reports*, **36**, 2044; *Chem. Abstracts*, **15**, 3664.
Johnson and Hooper (1921). *Public Health Reports*, **36**, 2037; *Chem. Abstracts*, **15**, 3664.
Osborne and Mendel (1911). *Carnegie Institution of Washington, Publication* 156, pt. ii, 83.
—— —— (1918). *J. Biol. Chem.* **34**, 537.
—— —— (1919). *J. Biol. Chem.* **37**, 572.
—— —— (1920). *J. Biol. Chem.* **41**, 515.
Osborne and Wakeman (1919). *J. Biol. Chem.* **40**, 383.

XXVI. THE ESTIMATION OF NON-PROTEIN NITROGEN IN BLOOD.

By ERIC PONDER.

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(*Received February 16th, 1922.*)

The object of this paper is to describe a rapid micro-method for the estimation of non-protein nitrogen in blood. Several methods have hitherto been described, but, for various reasons, are unsatisfactory. The method described by Cole [1919] is very lengthy, and requires 5 cc. of blood—an amount which cannot be obtained from small animals, and only with some inconvenience from man. The method of Folin and Denis [1916] is even more unsatisfactory. Several cc. of blood are required. The digestion mixture does not appear to be a suitable one, the contents of the incineration tube turning solid before the incineration is complete, and being afterwards quite insoluble in water: this defect is apparently due to an excess of phosphoric acid. Even if this occurrence be avoided, Nesslerisation is very difficult: Cole has observed this, and notes that a cloud rapidly appears. Frequently a brick-red precipitate appears also.

The following method, which may be applied to small quantities of blood, obviates these difficulties, and may be used with success when estimating the non-protein nitrogen in the blood of small animals.

Special apparatus etc. required:

1. The solutions required for the preparation of blood filtrates, as described by Folin [1919].

2. A digestion mixture. To 50 cc. of a 5 % copper sulphate solution add 100 cc. of 85 % phosphoric acid, and 300 cc. of pure concentrated sulphuric acid.

3. A boiling tube, 10 × 1 cm. The tube need not be of special glass. It is graduated at 3·5 cc. It is provided with a small loose stopper, shaped like a specific gravity bulb, which is introduced neck downwards into the boiling tube, so as to close the orifice.

4. A micro-filtration apparatus, such as is described in the paper dealing with the author's method for the estimation of blood-sugar [Ponder and Howie, 1921]. This is essentially a small filter which filters under slight suction.

5. Small calibrated pipettes, to deliver 0·2, 0·15, 0·5 and 1·5 cc.

6. Standard ammonium sulphate solution, containing 0·4716 g. of the purified salt per litre; Nessler's reagent, as described by Folin or Cole.

The estimation is performed as follows:

1. Blood is drawn from the finger, or from an animal's vein, into a 0·2 cc. pipette. The contents of the pipette are added to 1 cc. of distilled water:

the pipette is twice filled with water, and these volumes, carrying with them all traces of blood from the walls of the pipette, are added to the tube containing the blood and distilled water. 0·2 cc. of sodium tungstate and 0·2 cc. of 2/3 *N* sulphuric acid are added: the tube is allowed to stand for five minutes at least, its contents being occasionally shaken.

2. A filtrate is produced from the contents of the tube by about two minutes filtration in the micro-filter.

3. 0·5 cc. of filtrate is placed in the boiling tube, and to it is added 0·2 cc. of the digestion mixture diluted 1 in 4. The contents are boiled over a micro-burner, very gently. As soon as boiling occurs, the stopper is placed in the mouth of the tube. The boiling is continued for two minutes. The flame should be very low; bumping is thus prevented. At the end of the incineration, the flame is removed, and a few drops of water are added. The tube is allowed to cool, and is then filled to the mark with distilled water.

4. A standard is prepared. Place 3·15 cc. of distilled water in a tube, add 0·15 cc. of standard ammonium sulphate solution, and 0·2 cc. of the diluted digestion mixture.

5. Nesslerise the contents of the boiling tube and the standard simultaneously, by adding 1·5 cc. of Nessler's reagent. There is no difficulty in obtaining a clear yellow solution.

6. The solutions are matched in the colorimeter in the usual way. If the standard be set at 15, the non-protein nitrogen in 100 cc. of blood is 15 divided by the reading, and multiplied by 30.

It has not been possible, for the reasons given above, to compare this method with that of Folin and Denis. The method has however been applied to nitrogenous substances, of known nitrogen content, which require incineration before producing a colour with Nessler's reagent, and has been found accurate and satisfactory, the results obtained by the method and the calculated results agreeing closely.

Some results obtained in the examination of blood of various animals are given in the following table:

Animal	mg. in 100 cc.	Animal	mg. in 100 cc.
Man	36	Rabbit	43
,,	25	Frog	93
Cat	67	Lizard	76

In the case of the frog and lizard, the blood was obtained from the heart.

It is claimed for this method that (1) a very small quantity of blood is required, (2) that the technique is simple and rapid, (3) that the final Nesslerisation presents no difficulty, and (4) that the results given are accurate.

REFERENCES.

Cole, S. W. (1919). Practical Physiological Chemistry, 260.
Folin, O. (1919). A Laboratory Manual of Biological Chemistry, 179.
Folin and Denis (1916). *J. Biol. Chem.* **26**, 491.
Ponder and Howie (1921). *Biochem. J.* **15**, 171.

XXVII. THE CONDITIONS INFLUENCING THE FORMATION OF FAT BY THE YEAST CELL.

By IDA SMEDLEY MACLEAN.

(*Received February 22nd, 1922.*)

Very little is known of the story of fat metabolism in the lower organisms although a considerable amount of work has been carried out on the conditions attending the formation of fat in yeast. Yeast offers a particularly favourable field of study as it can readily be grown in large quantity and considerable variations can be effected in the conditions of its growth. In spite of the work that has been done but little progress has been made since the work of Naegeli and Loew [1878, 1879] carried out more than forty years ago.

The conclusions arrived at by Naegeli and Loew were briefly as follows:

(1) The fatty acid of the yeast cell consisted chiefly of oleic acid.

(2) The amount of fat obtainable from yeast was about 1 to 2 % if the dried yeast was directly extracted by ether; this figure might be raised to about 5 % by first evaporating the yeast with concentrated HCl several times on a water-bath. The acid destroyed the cell wall and the ether then was no longer prevented from extracting the fat by the impermeability of the cell wall.

(3) The more vigorous the growth of the yeast cell, the greater was the amount of fat formed. Both the total amount of dry substance formed and the percentage of fat it contained were raised.

(4) Other conditions being similar, the percentage of fat formed increased with the supply of oxygen. They found that the fat content of a yeast grown in a solution of sugar containing ammonium tartrate (2 %) which was aerated throughout the time of growth was 12·5 %, whereas a yeast grown on peptone and sugar at a low temperature with scanty respiration contained only 5 % of fat.

(5) Naegeli discussed the question of the origin of the fat formed and concluded that under different conditions both carbohydrate and protein might act as sources of fat. He drew attention to the rape seed which, before maturity, is filled with starch grains and from which, when ripe, the oil is pressed and to the case of fungi in which, when put into water, the plasma diminishes with the appearance of fat, the cellulose membrane also increasing during fat formation.

(6) Naegeli recognised also that the fat contained a sterol which he termed cholesterol and further that the conditions which led to an increase of fat led also to an increase of sterol.

The nature of yeast fat.

This has since been elucidated by the work of Hinsberg and Roos [1903, 1904], Neville [1913], Gérard [1895], and Smedley MacLean and Thomas [1920]. As the result of these investigations it has been shown that the fatty acids present are palmitic, oleic and linoleic with a small quantity of lauric. The fat is chiefly remarkable for the large proportion of sterol which it contains, the sterol being apparently identical with ergosterol, a characteristic constituent of the fats of all the lower plant world.

The amount of fat present in yeast.

In the normal yeast cell the percentage of fat present has been described as from 1 to 5 % of the dried yeast. Naegeli pointed out that the extraction of the air-dried yeast with ether only removed part of the fat, and that if the cells were first treated with concentrated HCl from two to three times as much fat could be extracted, this being however hydrolysed to the free fatty acids. Naegeli's results have been criticised by later observers who consider that prolonged treatment with the strong acid may give rise to ether-soluble decomposition products of the yeast cell and that the higher figures obtained do not represent the true percentage of fat.

Hinsberg and Roos [1903] and Bokorny [1916, 3] therefore retain the ether extraction method in determining the fat percentage of dried yeast. Bokorny [1916, 2] indeed did not confirm Naegeli's results; he treated the air-dried yeast for 24 hours with concentrated HCl and found that the percentage of fat was only 0·66 % of the weight of dried yeast compared with 2·66 % of fat obtained by ether extraction without the preliminary treatment with acid: the sticky mess obtained by treating the yeast with acid, he found unsuitable for ether extraction.

In a series of experiments carried out with the object of determining the proportion of fat to carbohydrate in the yeast cell under different conditions, I adopted the method of hydrolysing the yeast by boiling with *N* HCl for two hours, filtering and washing the residue with water until the washings no longer reduced Fehling's solution; the residue was then air-dried overnight at the laboratory temperature and extracted with ether in a Soxhlet apparatus and the filtrate and washings used for the estimation of carbohydrate. After evaporating off the ether, the residual fat was taken up with dry ether and dried to constant weight in a vacuum desiccator at the laboratory temperature.

I found that the amount of fat found in this way might be several times as great as the amount obtained by the direct extraction of the dried yeast with ether. In the latter method the yeast was dried by treating it with a large volume of absolute alcohol, the alcoholic extract was evaporated and the residue added to the dried yeast before extracting it with ether.

A comparison of the fat obtained by the two methods showed that the two specimens were similar in appearance and from both sterol separated on

standing; the iodine values of both varied considerably in different experiments but no consistent difference could be detected: the difference between the Wijs and Hubl numbers which may be taken as an indication of the amount of sterol present [Smedley MacLean and Thomas, 1921] also showed no constant variation between the two series of experiments.

Table I. *Showing the amount of fat extracted by ether before and after hydrolysis of the yeast.*

Sample of yeast	Weight of dried yeast g.	Amount of fat (*a*) Without hydrolysis: Weight g.	(*a*) Without hydrolysis: % on dry yeast	(*b*) After hydrolysis: Weight g.	(*b*) After hydrolysis: % on dry yeast
Pressed yeast (12·5 g.)	2·99	0·1118	3·74	—	—
	3·07	—	—	0·1948	6·35
	3·05	—	—	0·1892	6·20
12·5 g. above sample incubated 48 hours at 26° in glucose solution					
Oxygenated	4·17	0·0960	2·29	—	—
"	4·80	—	—	0·5402	11·24
Not oxygenated	4·18	0·1114	2·67	—	—
" "	4·80	—	—	0·2434	5·07
A pure culture of yeast grown on wort containing lactic acid (*N*/10) at 30°					
Oxygenated	8·05	0·1076	1·34	—	—
"	8·05	—	—	0·1975	2·45
Not oxygenated	6·49	0·0996	1·53	—	—
" "	6·49	—	—	0·1524	2·30

It must be admitted therefore that ether extraction of the yeast dried either in air or by means of alcohol, does not remove all the fat from the cells. The criticisms brought forward against Naegeli and Loew's method of repeated evaporation of the yeast with concentrated HCl cannot be urged against the much less drastic treatment of boiling for two hours with 3·6 % or even with 1·8 % HCl, a method by which the fat is not hydrolysed, its acid value being barely affected.

The state in which the fat occurs in the yeast cell.

Naegeli and Loew apparently regarded the hydrochloric acid as acting by impairing the cell membrane and thus permitting the entrance of the solvent into the cell containing the fat. They considered it probable that continued treatment with alcohol or ether would completely remove the fat, an expectation which does not appear to be realised even when the extraction is continued for a very long time. Two views present themselves: (1) a proportion of the fat may exist in the free state in the cell, being probably formed as a decomposition product of the cell-plasma. The remainder of the fat may be in combination in the plasma of the cell and may only be liberated on hydrolysis when some complex substance in the plasma is itself decomposed. (2) The sub-microscopic fat particles may be retained in a protein meshwork, only the larger fat particles being extracted by the ether. The smaller fat particles would then only

be liberated by the breaking down of the protein when they would be extracted by the ether.

All the known facts as to the extraction of fat from yeast are in agreement with the hypothesis that the fat is closely associated with the sterol and protein and possibly with the carbohydrate of the cell; this association may be of the nature of a chemical combination.

The evidence upon which this view is based, is as follows:

(*a*) Extraction with alcohol and ether removed readily from 1 to 3 % of yeast fat, calculated on the dried yeast, after which only traces of fat were obtained by long continued extraction.

(*b*) As stated above about twice as much fat may be removed from the yeast cell after boiling with normal or semi-normal acid as is obtained by direct ether extraction of the dried yeast. The greater part of the fat which is obtainable from dried yeast by direct extraction with ether is removed in a comparatively short time; further prolonged extraction only results in the separation of traces of fat.

Thus in an experiment carried out by Miss D. Hoffert, 12·5 g. yeast were soaked overnight in alcohol, the alcohol evaporated, the residue added to the yeast, the whole dried overnight and extracted with ether in 14 hours; the amounts of fat extracted varied from 0·0822 to 0·1060 g. 12·5 g. of the same sample of yeast were hydrolysed with *N* HCl for two hours, the solid residue dried overnight and extracted for 14 hours with ether; 0·2111 g. fat was obtained.

The amounts of fat obtained per hour by direct extraction with ether are shown in the following table.

Table II.

Time in hours...	1st	2nd	3rd	4th	5th	6th	7th	7th to 14th	Total in 14 hours
(1) Weight of fat from 12·5 g. yeast	0·0334	0·0216	0·0146	0·0090	0·0082	0·0079	0·0055	0·0058	0·1060
(2) „ „	0·0652	0·0077	0·0023	0·0013	0·0014	0·0013	0·0011	0·0019	0·0822
(3) „ „	—	—	—	—	—	—	—	—	0·0888

(*c*) Old yeast cells or cells grown under unfavourable conditions, *e.g.* an abnormally low or high temperature, give considerably higher fat percentages than normal cells when extracted directly with alcohol and ether. Such cells when examined microscopically show small globules of fat staining with osmic acid. In experiments where the period of incubation is long, partial autolysis of the yeast probably takes place—the amount of yeast formed is much reduced and the proportion of fat extracted by ether is greater. The total amount of fat obtained is decreased, but since the total amount of yeast is proportionately still less, the percentage of fat is raised.

Tubes containing 10 cc. wort were inoculated with a pure culture of brewer's' yeast and after 48 hours the contents added to 1500 cc. sterilised wort and incubated. The yeast was centrifuged, filtered and dried with

alcohol; the residue from the alcohol was added to the dried yeast and the product extracted with ether in a Soxhlet apparatus for 14 hours. From the figures given below it will be noted that (1) the quantity of yeast formed is in inverse ratio to the fat percentage, (2) the percentage of fat tends to be higher when the time of incubation is long and (3) incubation in the presence of 1 % lactic acid at 35° is particularly unfavourable and leads to the production of the smallest amount of yeast and the highest percentage of fat.

Table III. *Showing that a higher percentage of fat is extracted by ether from yeast which has been grown under unfavourable conditions.*

			Temperature 25–26°				
	(*a*) Reaction neutral			(*b*) 1 % lactic acid added			
No. days	Weight fat. g.	Weight dry yeast. g.	Fat %	No. days	Weight fat. g.	Weight dry yeast. g.	Fat %
2	0·105	7·4	1·4	2	0·131	6·5	2·0
2	0·205	8·9	2·3	2	0·117	5·3	2·2
3	0·143	7·7	1·8	2	0·110	5·0	2·2
3	0·152	7·4	2·0	3	0·168	5·8	2·8
3	0·155	6·5	2·4	3	0·130	5·8	2·2
6	0·155	4·6	3·3	5	0·149	4·2	3·6
				6	0·153	3·7	3·3
Mean...	0·153	7·1	2·2		0·134	5·2	2·6
			Temperature 35–36°				
4	0·127	4·1	3·1	4	0·148	3·1	4·6
4	0·101	2·1	4·7	4	0·063	2·6	2·4
4	0·105	2·9	3·5	4	0·076	1·3	5·2
6	0·083	2·4	3·4	8	0·087	0·96	9·0
8	0·051	2·9	1·7	12	0·032	0·49	6·5
12	0·058	1·5	3·8	12	0·028	0·69	4·0
Mean...	0·087	2·65	3·4		0·072	1·52	5·3

(*d*) Bokorny [1916, 3] found that when yeast is submitted to the action of protoplasmic poisons the amount of fat obtained by extraction was very considerably increased. He soaked pressed yeast for some hours in such solutions as phenol (5 %), formaldehyde, mercuric chloride, etc. While it seems unlikely that living processes would continue to be carried on by yeast soaked in 5 % phenol solution, it is certainly conceivable that such treatment might decompose the complex substance in the plasma and liberate fat from it, if fat be indeed one of its constituents.

Bokorny's experiments were carried out on small amounts of yeast and the quantities of fat weighed were small. It is known that protein readily absorbs phenol [Cooper, 1912] and it is possible that when the phenol-treated yeast was extracted with ether, the small amount of fat present may have been augmented by traces of phenol which contributed to the 12 % of fat obtained. In repeating this work it was found very difficult completely to remove the phenol, but I think there is no doubt that after the soaking with phenol the proportion of fat extracted by ether is appreciably increased. Bokorny regarded the increase as being caused by an abnormal secretion of fat deposited as a protection against unfavourable conditions of growth. More probably it is to be regarded as fat liberated from combination in the cell

contents by the action of the poison. It is interesting to note that a German patent (D.R.P. 309,266) recommends the auto-digestion of the yeast to ensure the liberation of the fat globules from the cells before extracting with solvents.

Conditions affecting the amount of fat in the cell.

During the Great War the question of using the lower organisms as sources of fat became one of practical importance especially in Germany where a good deal of work was carried out on these lines. Lindner's [1916, 1919] "mineral yeast" (*Endomyces vernalis*) was cultivated as a source of both fat and protein and in this organism a fat percentage of 18 % was claimed. The work of Bokorny and other observers was directed to producing a similar result with yeast; Bokorny [1916, 1] found that by using peptone as his nitrogenous food and repeatedly adding sugar, the fat content of yeast could be raised. In one German patent (D.R.P. 320,560) it is claimed that by applying the methods used by Lindner to increase the fat content of mineral yeast, the fat content of beer yeast and of pressed yeast may be raised to from 20 to 50 % viz. by growing a surface culture on a non-nitrogenous medium. The method of estimating the fat is not given and only the microscopic appearance is described. Another patent (D.R.P. 307,789) describes the application of hydrogen peroxide and of violent aeration of the glucose solution to increase the fat content of yeasts which do not form surface cultures. Here the increase of fat is not stated nor is the method of extraction indicated.

In the first series of experiments I carried out, the air-dried yeast was extracted with alcohol and ether. Pure cultures of yeast were grown on wort with and without aeration of the medium; pressed yeast was added to glucose solutions and to wort and incubated with and without aeration of the medium. No marked differences were produced in the amount of fat extracted and the oxygenation of the medium appeared to be without result. If however the yeast was first hydrolysed with normal HCl in the manner already described and the solid residue extracted with ether, very marked variations were observed and the fat content appeared to be largely raised by the aeration of the medium.

Pressed yeast incubated for 44 hours in glucose solution without oxygenation showed a marked increase in the total weight of the dried yeast, and the total weight of fat present was increased although the percentage of fat calculated on the dry weight was decreased. But in the oxygenated glucose solution, not only was the total dry weight of the yeast increased, but the percentage of fat was sometimes more than doubled. Thus in one experiment the fat percentage rose from 6·0 to 11·6 %; but while 11·6 % of fat was extracted after hydrolysis, part of the same material treated with alcohol and then extracted with ether showed only 3 % of fat. The increased amount of fat formed when yeast is grown in a glucose solution which is oxygenated throughout the experiment appears to be held in combination in the cell

plasma and is not extracted by ether until by hydrolysis it is set free from the cell complex. The ether-soluble material which was weighed as fat contained both fat and sterol.

Yeast incubated in a solution of glucose to which nitrogenous material has not been added is characterised by a high percentage of carbohydrate. If a given quantity of yeast be incubated for 44 hours in (1) a solution of glucose and in (2) wort containing the same percentage of carbohydrate, the total amount of yeast formed in the wort is from two to three times as much as in the carbohydrate solution. The total amounts of carbohydrate contained in the yeasts after incubation in (1) and in (2) respectively are approximately the same, though, since much more yeast is formed during the incubation in the wort, the *percentage* of carbohydrate in the latter specimen is much less. The total amount of fat as well as the percentage are considerably higher in the yeast incubated in the glucose solution.

It is not clear whether the decreased amount of fat in the yeast incubated in the medium rich in nitrogen is due to a lessened synthesis of fat or to an increased breaking down of the fat after it has been formed. The amount of fat is however markedly greater in the yeast from the oxygenated wort than in that from the wort which has not been aerated.

The part played by the oxygen requires further elucidation and is at present being further studied; it is uncertain whether, as Slator [1921] suggested in his study of the conditions affecting the growth of yeast, it acts by removing the detrimental influence of the carbon dioxide or by some specific action of its own. In all the experiments which I have so far carried out an increase in the total amount of fat has been associated with a high percentage of carbohydrate in the cell.

The observation of Naegeli that the more vigorous the growth of the yeast cell (as is the case in the aerated medium) the greater the amount of fat formed is therefore confirmed. It will be remembered that Naegeli extracted the fat after previously warming the yeast with concentrated HCl. Bokorny's observation that in strongly growing yeast cells there is a diminished fat content probably depends on the fact that he extracted the dried yeast directly with ether, for when this method of extraction is used his results are in agreement with those quoted above [Bokorny, 1916, 2].

In the following experiments 12·5 g. of pressed yeast were incubated for 44 hours at 26° in 1500 cc. of the sterilised medium, in some of the experiments a current of oxygen being passed through the medium during the experiment. The yeast was then filtered and the total reducing substance determined after hydrolysis by Bertrand's method, the result being calculated as glucose. The figures given for fat refer to the total amount of material soluble in dry ether.

The nature of the substance from which fat is formed.

Naegeli and Loew [1879] appear to have recognised clearly that fat is formed in moulds and yeasts from substances existing in the plasma of the

cell; they noticed that as the fat globules appeared the plasma diminished, and argued therefore that the fat could not be derived from the nitrogen-free carbon compounds since these were only present in very small quantity in the cell contents. In this case therefore they claimed that the fat must have been formed from protein. When peptone was used as the source of nitrogen, they regarded the sugar or tartaric acid in the medium as the source of fat.

Table IV. *Showing the effect of oxygenation.*

Medium	Oxygen	Yeast dry weight. g.	Fat weight. g.	Fat %	Carbohydrate weight. g.	Carbohydrate %	Nitrogen %
Original yeast		3·17	0·1643	5·2	0·9	28·2	—
		3·19	0·1812	5·7	—	—	—
In water	−	2·64	0·1734	6·2	0·55	20·8	—
„ „	+	2·65	0·1343	5·1	0·55	20·6	—
„ glucose	−	3·72	0·1892	5·1	2·0	53·8	—
„ „	+	4·17	0·4532	10·8	1·85	44·1	—
Original yeast		3·40	0·2277	6·7	0·92	27·2	—
		3·48	0·2158	6·4	0·88	26·0	—
In water	−	2·87	0·1542	5·4	0·66	23·0	—
„ „	+	3·03	0·1812	6·0	0·64	21·2	—
„ glucose	−	4·98	0·2824	5·7	2·50	50·1	—
„ „	+	5·69	0·6623	11·6	2·64	46·3	—
Original yeast		3·13	0·1892	6·05	0·49	15·7	7·87
		3·07	0·1948	6·37	0·55	17·9	7·67
In glucose	−	4·80	0·2440	5·07	2·40	50·0	5·10
„ „	+	4·80	0·5402	11·24	2·21	46·1	4·73
Original yeast	−	2·98	0·2183	7·32	0·51	17·2	8·78
		2·90	0·2193	7·56	0·52	18·1	8·69
In glucose	+	4·65	0·6267	12·43	2·00	23·3	4·77
Original yeast		3·93	0·2481	6·30	0·84	21·5	7·96
In wort	−	11·26	0·1637	1·45	2·42	21·5	8·55
„ „	−	12·27	0·2153	1·76	2·56	20·9	8·72
„ „	+	11·73	0·2846	2·43	2·63	21·1	8·51
Original yeast							
(1) In wort	−	6·44	0·1487	2·31	1·72	26·7	8·70
(2) „ „	−	3·29	0·1169	3·51	1·05	28·4	8·97
(1) „ „	+	10·04	0·4165	4·15	2·61	26·0	8·67
(2) „ „	+	9·27	0·3535	3·65	2·30	23·72	—

(1) Instead of 12·5 g., 2·5 g. of yeast containing 0·0505 g. fat was added to the solution.
(2) The inoculating dose was here 0·25 g. containing 0·005 g. fat.

They thought it probable that all materials for fat formation were first built into the protoplasm protein and in this way utilised for the formation of fat, the fat being subsequently split off and stored as reserve material.

The figures already quoted (Table IV) show conclusively that when yeast is grown in oxygenated glucose solution, where no external supply of nitrogen is available, the loss in protein even if the protein were wholly converted to fat could account only for a small portion of the fat formed. As it is extremely unlikely that all the amino-groups present would be converted into fatty chains, it is clear that some other source must be found for the manufacture of the fat. When yeast is grown in glucose solution there is a large accumulation of carbohydrate in the cell and since the protein which has disappeared is insufficient to account for the increase in fat, either the carbohydrate of the

external medium or that of the yeast cell itself must have acted as the starting material for the formation of fat. The following figures will make this apparent.

	Dry yeast g.	Carbohydrate g.	Fat g.	Nitrogen g.	Protein g.
1. 12·5 g. of the original sample of yeast contained	3·1	0·52	0·192	0·2409	1·50
12·5 g. yeast incubated 44 hours in oxygenated glucose solution at 26° and filtered. The residue contained	4·80	2·21	0·540	0·2270	1·42
Gain or loss	+1·7	+1·69	+0·348		−0·08
2. 12·5 g. of the original sample of yeast contained	2·94	0·52	0·219	0·257	1·61
After incubating 44 hours at 26° in glucose solution and filtering. The residue contained ...	4·65	2·00	0·627	0·222	1·39
Gain or loss	+1·71	+1·48	+0·408		−0·22

I endeavoured to find out whether the carbohydrate stored in the yeast cell was converted in the presence of a free supply of oxygen into fat; a sample of yeast which had been grown in glucose solution for 44 hours and contained a high carbohydrate percentage (46·7 %) was then transferred to water and a rapid current of oxygen passed through for about 20 hours. The carbohydrate and fat were determined in the yeast before and after it was submitted to the action of the oxygen.

3·84 g. dry yeast contained before being submitted to the action of oxygen 0·1932 g. fat and 1·79 g. carbohydrate; afterwards 0·2146 g. fat and 0·96 g. carbohydrate were detected. In another experiment 3·32 g. dry yeast contained 0·1477 g. fat and 1·446 g. carbohydrate, after growing in the glucose solution. After oxygenation in a solution containing 1 % of potassium and magnesium phosphates 0·1736 g. fat and 0·88 g. carbohydrate were found. Though the differences in the amounts of fat are not very large the increase suggests that the fat is formed from carbohydrate material stored inside the yeast cell.

Conclusions.

Free fat exists in the normal yeast cell in small amount; the percentage of fat is increased in old and degenerating cells, in cells grown under unfavourable conditions and in cells exposed to the action of protoplasmic poisons. A large part of the fat normally present in yeast and of the sterol associated with it, is in some form of combination in the plasma of the cell and is not extracted from it by treatment with alcohol and ether. This complex is decomposed by boiling with dilute mineral acids and the fat can then be readily extracted from it by ether. It is possible that part of the protein and carbohydrate which are hydrolysed by the dilute acid are also combined with the sterol and fat.

A free supply of oxygen and a non-nitrogenous medium rich in carbohydrate are conditions producing an increased amount of fat in yeast but

the fat formed in this way appears to be entirely held in combination in the yeast cell, and is only set free by hydrolysis.

The fat and sterol formed in this way are derived from carbohydrate.

This investigation has been carried out for the Food Investigation Board of the Department of Scientific and Industrial Research, and I desire to express my thanks for the grants which have been received for this purpose. I should like also to thank Miss Florence Banks for the assistance she has given in the experimental part of the work.

REFERENCES.

Bokorny (1916, 1). *Allgemein. Brauer- und Hopfen-Zeitung*, **56**, 603.
—— (1916, 2). *Allgemein. Brauer- und Hopfen-Zeitung*, **56**, 1479.
—— (1916, 3). *Biochem. Zeitsch.* **75**, 346.
Cooper (1912). *Biochem. J.* **6**, 362.
Gérard (1895). *Compt. Rend.* **126**, 909.
Hinsberg and Roos (1903). *Zeitsch. physiol. Chem.* **38**, 1.
—— —— (1904). *Zeitsch. physiol. Chem.* **42**, 189.
Lindner (1916). *Wochsch. Brauerei*, **33**, 193.
—— (1919). *Zeitsch. techn. Biol.* **7**, 79.
Naegeli and Loew (1878). *Annalen*, **193**, 322.
—— —— (1879). *München Akad. Sitzber.* 289.
Neville (1913). *Biochem. J.* **7**, 347.
Slator (1921). *J. Chem. Soc.* **119**, 115.
Smedley MacLean and Thomas (1920). *Biochem. J.* **14**, 483.
—— —— (1921). *Biochem. J.* **15**, 319.

XXVIII. THE ACTION OF WHOLE BLOOD UPON ACIDS.

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Although very many investigations have been made upon the alkali reserve of serum and plasma, and upon the movements of ions which take place when red corpuscles are exposed to different tensions of CO_2, it seems that no quantitative study of the acid-neutralising power of whole blood has been made. It is commonly assumed and stated that whole blood is more perfectly buffered than plasma. There is no difficulty in investigating this matter if the blood is placed in an isotonic medium.

Method.

Whole blood was shaken with 0·01*N* H_2SO_4 containing 0·9 % NaCl. The mixture was then centrifuged and the supernatant fluid titrated to ascertain how much of the acid had been neutralised, and examined by the electrode for H-ion concentration. The number of volumes of 0·01*N* acid added to one volume of blood varied from 2·28 to 9·66; the actual amounts used are given in the tables. The mixture of blood and acid was in all cases made up with 0·9 % NaCl to 8·25 cc. as this volume gave a convenient amount for duplicate titrations and H-ion determination. It was then shaken vigorously for five minutes in a wide centrifuge tube in a strong current of CO_2-free air to remove CO_2, and centrifuged in corked tubes. Of the supernatant fluid duplicate portions of 2·5 cc. were taken, mixed in test tubes with 5 cc. freshly boiled distilled water and nine drops of 0·1 % phenol red in alcohol, and titrated with 0·01*N* NaOH in a comparator, a phosphate solution of p_H 7·4 being used as colour standard. The remainder of the supernatant fluid was used for H-ion determination. Plasma was treated in exactly the same way as the whole blood except that the centrifuge was not used. Two series of observations were made, namely upon human blood derived from one healthy subject over a period of about three months, and upon guinea-pig's blood (Tables I and II, and Figs. 1 and 2).

It is at once evident in Fig. 1 that the behaviour of whole blood is quite different from that of plasma. However much acid is added to the human

plasma, roughly the same amount is neutralised; in fact plasma behaves, though with much wider variations, like a solution of caustic soda. With whole blood on the other hand the amount of acid neutralised increases with the amount added in such a way that round about 80 % of the acid is neutralised in every case. The exact percentages can be seen in Table I; they range from 89 to 78 %, and tend to diminish as the amount of acid increases.

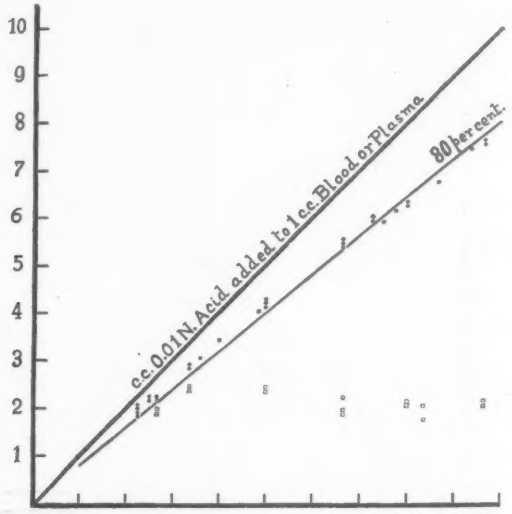

Fig. 1. NEUTRALISATION OF ACID BY HUMAN WHOLE BLOOD AND PLASMA. The points of intersection of the diagonal line with the ordinates represent amounts of acid added to 1 cc. blood or plasma; the points marked ● and □ on these ordinates represent the fractions of these amounts which are neutralised by the blood (●) or plasma (□). A second oblique line has been drawn at a height which is 80 % of the height of the diagonal.

The results obtained with the whole blood of guinea-pigs (Fig. 2) are confirmatory; the amounts neutralised keep rather closer to the 80 % line. With the plasma, however, extremely wide variations were found; in some cases the plasma had scarcely any neutralising power. This is probably due to the disturbances of circulation and respiration brought about in the taking of the blood, which are quite absent when blood is drawn from the human subject. It is remarkable that there is no indication in the results obtained from whole blood of these variations in the plasma. The maximum amount of 0·01*N* acid neutralised by 1 cc. plasma is about the same (2·3 to 2·4 cc.) in both cases (man and guinea-pig) and indicates that the "alkali reserve" of the plasma is equivalent to that of a 0·024*N* solution.

The addition of increasing amounts of acid to whole blood cannot of course be carried on indefinitely, because haemolysis supervenes. This occurs in human blood when more than about 11 cc. 0·01*N* acid is added to 1 cc. blood, whereas 1 cc. guinea-pig's blood is lysed by 7 or 8 cc. 0·01*N* acid. The amounts

of acid used in these experiments are not so large as to be altogether beyond the range of what may occur in the body; thus in diabetic coma the concentration of compounds of aceto-acetic and β-hydroxybutyric acids in the blood may be that of a 0·03N solution [Kennaway, 1918]. This would be in some degree comparable with an experiment in which 3 cc. 0·01N acid was added to 1 cc. blood (see Figs. 1 and 2). No methhaemoglobin was detected with the spectroscope in these mixtures even when more than enough acid to produce lysis was added.

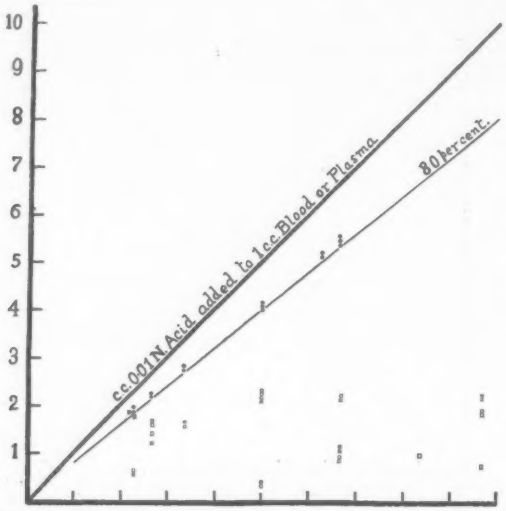

Fig. 2. NEUTRALISATION OF ACID BY GUINEA-PIG'S WHOLE BLOOD AND PLASMA. For description, see Fig. 1.

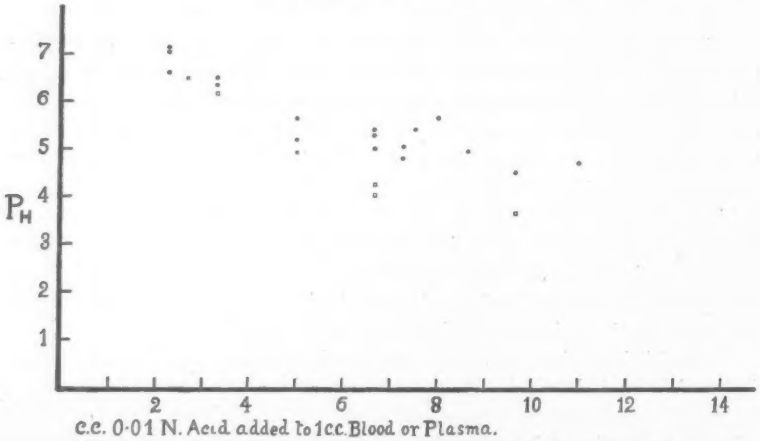

Fig. 3. p_H OF MIXTURES OF ACID AND HUMAN BLOOD (●) OR PLASMA (□).

Table I (see Figs. 1 and 2). *Neutralisation of acid by whole blood.*

All mixtures of blood and acid were made up to 8·25 cc. with 0·9 % NaCl.

Mixture cc.				Human blood			Guinea-pig's blood		
Blood	+ Acid		Ratio cc. 0·01*N* acid to 1 cc. blood	1 cc. blood neutralised cc. 0·01*N* acid	Percentage of acid neutralised	Final p_H	1 cc. blood neutralised cc. 0·01*N* acid	Percentage of acid neutralised	Final p_H
	0·01*N*	0·005*N*							
1·75	4·0	—	2·286	1·83	80·2	7·05	1·906	83·4	—
,,	,,	—	,,	1·83	80·2	—	—	—	—
,,	,,	—	,,	1·94	85·0	7·1	—	—	—
,,	,,	—	,,	2·03	88·8	6·6	—	—	—
1·0	—	5·0	2·5	2·20	88·0	—	—	—	—
1·50	4·0	—	2·66	2·22	83·4	—	2·20	82·7	—
1·50	5·0	—	3·33	2·90	87·1	6·5	2·76	82·9	6·15
,,	,,	—	,,	2·84	85·3	—	2·84	85·4	—
,,	,,	—	,,	2·87	86·0	6·35	—	—	—
0·75	—	5·0	,,	2·83	85·0	—	—	—	—
1·0	—	7·25	3·6	3·04	84·4	—	—	—	—
0·75	—	6·0	4·0	3·39	84·8	—	—	—	—
0·75	—	7·25	4·8	4·05	84·3	—	—	—	—
1·0	5·0	—	5·0	4·15	83·0	5·2	4·01	80·2	5·3
,,	,,	—	,,	4·08	81·6	5·65	4·13	82·6	5·8
0·75	—	7·5	,,	4·25	85·0	—	—	—	—
0·8	5·0	—	6·25	—	—	—	5·14	82·2	5·55
0·75	5·0	—	6·66	5·35	80·3	5·4	5·52	82·9	5·05
,,	,,	—	,,	5·53	83·0	5·3	5·34	80·2	5·8
1·0	7·25	—	7·25	5·93	81·8	5·05	lysis	—	—
,,	,,	—	,,	6·01	82·5	4·8	—	—	—
0·5	—	7·75	7·75	6·16	79·5	—	—	—	—
0·75	6·0	—	8·0	6·28	78·5	5·65	—	—	—
0·75	6·5	—	8·66	6·79	78·4	4·95	—	—	—
0·75	7·0	—	9·33	7·48	80·1	—	—	—	—
0·75	7·25	—	9·66	7·55	78·2	4·5	—	—	—
,,	,,	—	,,	7·58	78·5	—	—	—	—
0·5	5·5	—	11·0	lysis	—	—	—	—	—

Table II (see Figs. 1 and 2). *Neutralisation of acid by plasma.*

Acid and plasma were mixed in the same amounts as those given in the first column of Table I for acid and whole blood.

	Human plasma			Guinea-pig's plasma		
Ratio cc. 0·01*N* acid to 1 cc. plasma	1 cc. plasma neutralised cc. 0·01*N* acid	Percentage of acid neutralised	Final p_H	1 cc. plasma neutralised cc. 0·01*N* acid	Percentage of acid neutralised	Final p_H
2·286	—	—	—	0·579	25·3	5·05
,,	—	—	—	1·797	78·6	6·4
,,	—	—	—	1·825	80·0	6·7
2·66	1·93	72·5	6·5	1·308	50·0	5·55
,,	2·25	84·6	—	1·59	60·0	6·6
3·33	2·41	72·4	6·15	1·595	47·7	5·8
5·0	2·38	47·6	—	0·347	7·0	4·2
,,	2·40	48·0	—	2·18	43·6	4·5
,,	—	—	—	2·31	46·2	4·35
6·66	1·915	28·8	4·0	0·84	12·6	4·0
,,	2·22	33·3	4·25	2·18	32·7	3·95
,,	—	—	—	1·10	16·5	4·1
8·0	2·06	25·8	—	—	—	—
8·33	1·88	22·6	—	0·96	11·6	—
9·66	2·09	21·6	3·65	0·73	7·6	3·45
,,	2·06	21·3	—	1·86	19·3	3·05
,,	2·11	21·8	—	2·16	22·4	3·4

The measurements of H-ion concentration (Fig. 3 and Tables I and II) show that the whole blood does not bring this concentration to any constant level when different amounts of acid are added. With the addition of increasing amounts the H-ion concentration increases at a diminishing rate, roughly from p_H 7 to 4. In striking contrast to their effects upon titratable acidity, the whole blood and the plasma have very much the same effect upon p_H; the plasma shows a rather greater acidity, as one would expect. The rather wide variations in p_H are no doubt due to the errors of pipette measurement, and they would be wider still if the mixtures were not buffered; thus an error of only 0·01 cc. in the pipetting of 0·01*N* acid in these experiments would, in an unbuffered solution, be nearly sufficient to produce the difference between p_H 5 and 6.

This quite unexpected behaviour of whole blood towards acids requires further investigation. The word "neutralisation" has been used above, for want of any less committal term, to denote simply a disappearance of titratable acid, which is all that is actually observed; this disappearance might be due to combination with inorganic or organic bases, or to some form of adsorption, or to both of these. The inorganic bases of the red corpuscles are insufficient to account for the neutralisation of these amounts of acid. 1 cc. of blood can neutralise 7·5 cc. 0·01*N* acid, of which 1·2 cc. is to be assigned to the plasma and 6·3 cc. to the corpuscles (see Tables I and II). The amounts of K and Na present, according to Schmidt's analyses, in 0·5 cc. of red corpuscles, are equivalent to about 5 cc. 0·01*N*. Hence these bases would be unable to neutralise such an amount of acid even if they were wholly combined with carbonic acid, whereas the analyses show that they must be combined chiefly as chlorides, phosphates, and sulphates.

The following points suggested themselves for investigation:

(*a*) Is the amount of acid neutralised dependent upon its dilution?

Fig. 4 and Table III show the amounts of acid neutralised when constant amounts of human blood (0·75 cc.) from the same subject and of 0·01*N* acid (5 cc.) were allowed to react in the presence of varying amounts of saline, the acid being diluted before the blood was added, and the total volume ranging from 6·25 to 45·75 cc.; the concentration of acid thus varied seven-fold. The differences observed in the case of any one of the four samples of blood examined thus are mostly within the range of experimental error. Hence, under the conditions studied, dilution does not have any distinct influence upon this neutralisation of acid.

(*b*) Is the acid taken up by the corpuscles liberated if they are re-suspended in a fresh neutral medium? This was found not to be the case. 1 cc. blood was shaken with from 5 to 7·5 cc. 0·01*N* acid in saline in the manner described under "Method" above, and centrifuged, and the supernatant fluid drawn off as completely as possible; the corpuscles were then re-suspended in neutral saline, shaken for five minutes, let stand for a time, the mixture then centrifuged and the supernatant fluid titrated. Only traces of acid (0·05 cc. 0·01*N*)

were recovered; such an amount might well have remained enclosed between the packed-down corpuscles when the first supernatant fluid was removed.

(*c*) Is the neutralisation of acid by whole blood dependent upon the structure of the red corpuscles, or does lysed blood act in the same way?

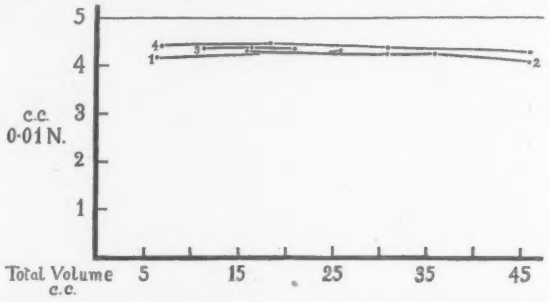

Fig. 4. NEUTRALISATION BY WHOLE BLOOD OF ACID IN DIFFERENT CONCENTRATIONS. 0·75 cc. blood was mixed with 5 cc. 0·01*N* acid diluted with saline. Points plotted represent the amount of acid neutralised. The numbers 1, 2, 3, 4 refer to four different samples of blood.

Table III (see Fig. 4). *Neutralisation by whole blood of acid in different concentrations.*

		0·75 cc. blood + 5 cc. 0·01*N* acid.			
Sample of blood	Total volume cc.	cc. 0·01*N* acid neutralised	Sample of blood	Total volume cc.	cc. 0·01*N* acid neutralised
1	6·25	4·17	3	11·25	4·37
	25·75	4·31		16·25	4·40
2	15·75	4·32		20·75	4·37
	30·75	4·24	4	6·75	4·41
	35·75	4·25		18·25	4·43
	45·75	4·05		30·75	4·37
				45·75	4·27

The difficulty in settling this question is due to the very deep colour of lysed blood; this necessitates considerable dilution before titration can be carried out. The small amount of acid to be titrated is in very dilute form and it is difficult to prevent the presence of appreciable quantities of CO_2 in the large volume of water required. It might be possible to employ dialysis, but it seemed that this might introduce various other sources of error. It may be best to describe one of the experiments in detail. The lysed blood was prepared by freezing and thawing four times. The following mixtures were made:

Blood +	{0·01*N* H_2SO_4 in normal saline}	+ Normal saline	Total volume	Burette reading cc. 0·01*N* NaOH
Unlysed {0·5	4	4	8·5	0·20, 0·21
{0·5	0	8	,,	0·11, 0·12
Lysed {0·5	4	4	,,	0·50, 0·50
{0·5	0	8	,,	0·115, 0·11

The mixtures were shaken for five minutes and centrifuged, in the ordinary way; the two with 8 cc. saline only and no acid served as "blanks." Duplicate portions of 1 cc. of each supernatant fluid were taken, diluted with 36 cc.

freshly boiled water and 2 cc. 0·1 % phenol red and titrated in the comparator; the colour in the lysed samples was very slight at this dilution (1 of blood in 663) but was matched by diluted blood placed in front of the colour standard. The results of three such experiments were as follows:

							% neutralised
I.	1 cc. unlysed blood	neutralises	6·21	cc. out of	8·0	cc. 0·01*N*	77·7
	1 cc. lysed	,,	1·54	,,	,,		19·2
II.	1 cc. unlysed	,,	6·28	,,	,,		78·5
	1 cc. lysed	,,	1·37	,,	,,		17·1
III.	1 cc. unlysed	,,	6·93	,,	,,		86·9
	1 cc. lysed	,,	2·31	,,	,,		29·0

As has been explained above, this form of experiment is not adapted to a high degree of accuracy; but it does show beyond doubt that lysed blood has only from one-quarter to one-third of the acid-neutralising power of unlysed blood. The actual difference measured in this comparison of lysed and unlysed blood is that between two burette readings; with the burette employed the difference of level was about $2\frac{1}{2}$ inches, a sufficiently obvious amount. The acid-neutralising power of lysed blood is not greater than that of plasma (see Table II). The remarkable property of whole blood which is illustrated in Figs. 1 and 2 seems then to be due to the structure or physical condition of the red corpuscles. Probably there are many such forms of adsorption or pseudo-adsorption which have not yet been described.

Summary.

The action of whole blood upon acids is quite different from that of plasma. If a series of amounts of sulphuric acid is added to plasma, and to whole blood, so as to produce p_H between 7 and 4, it is found that the amount of titratable acid removed by whole blood increases with the amount added in such a way that a roughly constant percentage, namely about 80 %, of the acid disappears; whereas the amount neutralised by plasma does not vary with the amount added.

It was not found possible to wash out the acid taken up by the red corpuscles. Within the range investigated, the amount of acid removed was independent of the dilution. The inorganic bases of the corpuscles are insufficient to neutralise the acid. Since lysed blood does not take up acid in this way it seems that the action of whole blood upon acids must be due to some form of adsorption which is dependent upon the structure or physical condition of the red corpuscles.

Reference.

Kennaway (1918). *Biochem. J.* **12**, 120.

XXIX. SALIVARY SECRETION IN INFANTS.

By CLEMENT NICORY.

(*Received February 28th, 1922.*)

THERE seems to be great difference of opinion as to the age of onset of the secretion of ptyalin in the saliva and its relation to the food of the child.

Schiffer [1891] says that the ptyalogenous function is present in the new-born child in the submaxillary gland, but is not established in the parotid under the age of two months.

Moll [1905] obtained saliva containing ptyalin from a parotid fistula in a seven months foetus.

Ibrahim [1908] confirmed the work of Moll, and found that ptyalin appeared earlier in the parotid than in the submaxillary gland. This seems natural when one remembers that the parotid is a purely serous gland, and most of the ptyalin in the mixed saliva of the adult comes from it as shown by Pavlov.

Berger [1899] examined the dry residue of the saliva in new born babies, and found that the foetal and adult salivary secretions do not differ very considerably in their consistency. The dry residue in new-born babies being 0·82 % and in adults about 0·79 %.

According to most investigators the secretion of saliva is very slight until after the sixth week of life when the amount increases considerably. Korowin succeeded in collecting as much as $1\frac{1}{4}$ cc. in five minutes in a child of four months.

Finzi [*Rev. Hyg.*] found that up to the age of six months the amylolytic power of the saliva was the same, whether the food contained starch or not, but after that age the power increased if the food contained starch.

Histologically the infant's salivary glands are relatively rich in connective tissue and in blood vessels, and the mucous cells are few in number. At two years of age however it is impossible to distinguish microscopically an infant's salivary gland from that of an adult.

In an extensive investigation of the subject I have recently obtained the following results at St Thomas's Hospital and the Evelina Hospital for Children, London.

The saliva was collected by giving infants plugs of gauze weighing 500 mg. to suck, the amount of saliva being estimated by the increase in weight of the plugs. The latter were extracted with 5 cc. of distilled water for one hour and then two series of tests were done.

(*a*) Equal parts of salivary extract and 1 % starch solution were mixed.
(*b*) To 1 cc. of the salivary extract 2 cc. of 1 % starch solution were added.
The test tubes were kept at a temperature of 40° C.
The following table shows some of the results obtained:

Age	Condition of infant	Nature of diet	Equal parts of starch solution and salivary extract	Two parts starch solution to one part salivary extract
34 weeks (premature)	—	Whey	98 minutes	130 minutes
36 „ „	—	„	30 „	104 „
36 „ „	Not as good as others	„	No change after two hours	
5 hours (after birth)	Fair	Breast	23 minutes	30 minutes
24 „	Good	„	16 „	30 „
24 „	Poor	„	29 „	41 „
48 „	Good	„	17 „	29 „
48 „	Very poor	„	No change after two hours	
12 days	Poor (one of twins)	Cows' milk and water	17 minutes	65 minutes
9 weeks	Fair	Breast	8 „	11 „
10 „	Good	„	4 „	5 „
11 „	Fair	„	7 „	8 „
3 months	Poor	Cows' milk	6 „	8 „
5 „	Very poor	Breast and bread	9 „	13 „
8 „	Fair	Breast	1 minute	3½ „
1 year	Good	—	42 seconds	1 minute

The time indicated in the table was taken at the moment when the mixture of enzyme and starch solution had reached the achromic point as indicated by the iodine test. At this point the starch has reached the stage of convertion into achroodextrin and some maltose. The presence of reducing sugar was demonstrated with Fehling's solution.

An analysis of the results obtained from the saliva of 80 different infants supported the following conclusions:

(1) Ptyalin is present in the saliva of infants at least 1½ months before term although only in small quantities.

(2) The amount of enzyme increases gradually to the age of one year, when the composition of the child's saliva becomes identical with that of the adult.

(3) At all ages the quantity of ptyalin present varies with the general condition of the child, being larger in strong than in weak infants.

Inquiries were made among the mothers attending the out-patient departments as to the nature of the diet administered to their infants, and one was surprised to find that a large number of them fed their babies on bread soaked in milk and ground rice at the early age of 11 weeks and even younger, and barley water was a diluent used by most of them. Quite a number of these children were in fair condition and suffering from no digestive trouble.

According to Halliburton [1913] and others no diastatic enzyme is present in the pancreatic juice of infants.

In view of the fact that an amylolytic enzyme is present in the saliva of babies in quite appreciable quantities, and that a number of infants fed on milk plus starchy foods manage to utilise a large quantity of the starch,

there seems every reason for assuming that diastase is present in the pancreatic secretion of infants.

At the commencement of my investigations it appeared as if children who had taken starch in their diet secreted more ptyalin than those who had had none, indicating a capacity of adaptation as postulated by Pavlov, but after examining a larger number it seemed that the presence of starch in the diet makes no appreciable difference in the quantity of ptyalin present in the saliva.

REFERENCES.

Berger (1899). *Inaug. Dissert.* St Petersburg.
Finzi. *Rev. Hyg. et Méd. Inf.* **8**, No. 3, 224.
Halliburton (1913). *Handbook of Physiology*, p. 520.
Ibrahim (1908). *Naturforsch. Vers. Köln.*
Moll (1905). *Monatschr. f. Kinderheilk.*
Schiffer, Korowin and Sweifel (1891). Cited in Halliburton's *Chemical Physiology.*

XXX. THE RELATION OF SALIVARY TO GASTRIC SECRETION.

By TOMOICHI NAKAGAWA (Osaka).

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(*Received March 1st, 1922.*)

Saliva, besides its main functions in assisting deglutition and exercising some amylolytic action, has other functions of secondary importance which also play a certain rôle in digestive processes. Maxwell [1915] found that boiled starch has a marked inhibitory action on peptic digestion, but that the products of amylolytic hydrolysis have not this effect. Thus the amylolytic ferment of the saliva acquires a new importance by removing the inhibitory factor and thus indirectly assisting in the gastric digestion. Maxwell, in all his experiments, used commercial pepsin. The object of the experiments described in this communication was to repeat Maxwell's experiments with natural gastric juice and to extend them to the rennet action of gastric juice.

A. Saliva and peptic digestion.

Pure gastric juice was collected by "sham feeding" from a dog which had a gastric fistula and an oesophagostomy. Fresh samples were used for each experiment. Mett's method was found to be sufficiently accurate for the purpose of these experiments and was used throughout the research. The Mett's tubes were left to digest for ten hours at 40°. All the experiments were repeated a considerable number of times, and those given in this communication serve only as examples.

Exp. 1. The seven small flasks containing the solutions named in Table I were kept at 40° for one hour; at the end of this time 5 cc. of gastric juice were added to each flask, and after another half hour the Mett's tubes were placed in each sample. In ten hours the readings of the digested tubes given in Table I were observed.

Table I.

		Digestion of Mett's tubes in mm.
1.	3 cc. of 2 % boiled potato starch + 1 cc. fresh human saliva	5·3
2.	" " " " " + " boiled " "	3·9
3.	" " raw " " + " " " "	5·4
4.	" " soluble starch + " " " "	5·3
5.	" " erythrodextrin + " " " "	5·2
6.	" " maltose + " " " "	5·4
7.	" " glucose + " " " "	5·5

The figure for flask 2 is the only one that shows a deviation from the rest. This flask was also the only one that contained the starch in a colloidal form. The hydrolysis of the starch in flask 1 was carried to the achromic point.

Exp. 2. In this experiment the actions of hydrolysed and unhydrolysed starches of different origin were compared. The different pairs given in Table II must not be compared with each other, because, although the hydrolysis was carried on for the same length of time, it reached a different stage with each starch as determined by the iodine reaction.

Table II.

	Digestion of Mett's tubes in mm.
1. Potato starch hydrolysed	5·3
„ „ unhydrolysed	3·9
2. Maize „ hydrolysed	3·0
„ „ unhydrolysed	2·2
3. Wheat „ hydrolysed	3·3
„ „ unhydrolysed	2·6
4. Rice „ hydrolysed	5·2
„ „ unhydrolysed	4·2

In each case 1 cc. of saliva, boiled or unboiled, was added to 3 cc. of the 2 % solution, kept in the thermostat for one hour, and then mixed with 5 cc. of gastric juice. The readings were taken at the end of ten hours.

Maxwell's observations made with commercial pepsin are extended in these experiments to normal gastric juice.

The theory of adsorption of the pepsin by the colloidal starch advanced by Maxwell seems to be the most probable explanation of the experiments described. There is one factor of general interest which should be mentioned. As has been described by several observers, the gastric juice secreted on a carbohydrate food is always the richest in pepsin. On the other hand, carbohydrates, when in a colloidal form, hinder peptic digestion. This seems to be another case of a delicate adaptation of the organism in providing more ferment when its action meets with difficulties.

B. Saliva and milk clotting.

We have seen that the carbohydrates in a colloidal form behave towards normal gastric juice and the commerical preparation of pepsin in the same manner. We now proceeded to determine whether the carbohydrates behave in the same way towards the rennet of gastric juice.

The experiments with milk clotting were performed in the same way as those with peptic digestion, but instead of the Mett's tubes, 5 cc. of cow's milk were added to each test tube and the time required for clotting noted.

The action of the boiled starch on milk clotting was found to be the opposite to its action on peptic digestion. In each experiment the addition of starch in a colloidal form produced a considerable acceleration of the clotting. Exp. 3 serves as an example.

Exp. 3. The seven test tubes containing the solutions were placed in a thermostat at 40°. After one hour 0·3 cc. of gastric juice was added to each test tube and after a further 30 minutes 5 cc. of cow's milk. The time of clotting of each sample is given in Table III.

Table III.

		Time of clotting in mins.
1.	3 cc. boiled potato starch + 1 cc. fresh human saliva	15
2.	„ „ „ „ + „ boiled „ „	3
3.	„ raw „ „ + „ „ „ „	15
4.	„ soluble starch + „ „ „ „	14
5.	„ erythrodextrin + „ „ „ „	14
6.	„ maltose + „ „ „ „	15
7.	„ glucose + „ „ „ „	16

As in the first experiment, the only figure which deviates from the others is the one corresponding to the test tube containing the starch in a colloidal form. But in this experiment the difference is in the opposite direction, showing a marked acceleration.

Exp. 4. Some other starches (beans, sago, barley, oats, etc.) were tried, and always with the result that the starch in a colloidal form produced an acceleration of milk clotting. Table IV gives some of the figures obtained.

Table IV.

	1 cc. saliva	Gastric juice	Milk cc.	Time of clotting mins.
3 cc. boiled potato starch	fresh	0·3	5	9
„ „ „ „	boiled	„	„	3
„ „ bean „	fresh	„	„	8
„ „ „ „	boiled	„	„	3
„ „ rice „	fresh	„	„	8
„ „ „ „	boiled	„	„	5
„ „ maize „	fresh	„	„	6
„ „ „ „	boiled	„	„	4

In all the above experiments human filtered saliva was used. The filtered saliva was quite watery and contained hardly any mucin as determined by precipitation with acetic acid. The result of the experiments with milk clotting indicates that the amylolytic action of saliva indirectly slows the clotting of the milk in the presence of colloidal starch. The experiments provide one more example of the difference in behaviour of pepsin and rennin.

Under normal conditions it is rather exceptional for saliva to have this effect on milk clotting, and it certainly never occurs in animals deprived of the salivary amylase. It is of some interest, however, that even in such animals (dog) a fair amount of saliva is secreted on milk although it is of no use for its deglutition and has no direct relation to its digestion. In the dog the submaxillary saliva secreted on milk is richer in mucin than the saliva secreted on any other food. In sucklings there is a profuse secretion of viscid saliva. These facts suggest that saliva itself, besides its indirect action described above, has some importance in the digestion of milk. Borisov [1914] has shown that in dogs with gastric fistula milk gives finer clots when mixed with saliva, and ascribes it to the physical action of the viscous saliva. Allaria [1912] found occasionally a slight slowing in milk clotting when mixed saliva from an infant was added.

The following experiments were performed in order to determine whether saliva has any direct effect on milk clotting.

Parotid and submaxillary saliva were collected from a dog having salivary

fistulae of the two glands. The saliva was diluted ten times with thrice distilled water. To each 5 cc. of the diluted saliva 5 cc. of milk were added and then mixed with 0·5 cc. of gastric juice. The time required for milk clotting in the thermostat at 40° is given below.

Table V.

		Time of clotting in mins.
1.	5 cc. tap water + 5 cc. milk	2
	„ ordinary distilled water + 5 cc. milk	2
	„ parotid saliva + 5 cc. milk	3
	„ submaxillary saliva + 5 cc. milk	6
2.	„ submaxillary saliva (10 times diluted) + 5 cc. milk	5
	„ tap water + 5 cc. milk	2
3.	„ pure submaxillary saliva (undiluted) + 5 cc. milk	∞
	„ tap water + 5 cc. milk	2

The sample of parotid saliva used in this experiment did not contain any mucin, which is often present in dog's parotid secretion.

Brennemann [1917] showed that in rapid clotting larger curds are formed than when the clotting of milk is slow. As the presence of the viscid saliva delays the clotting of the milk, this explains the observations of Borisov quoted above. It is certain that this effect of the submaxillary saliva does not depend on a change in the reaction. The addition of colloidal starch as used in the above experiments to acid solutions did not change their p_H as determined colorimetrically. Parotid saliva, being not less alkaline than submaxillary, has no such delaying effect on milk clotting. The action of the viscid saliva seems to be better explained by ascribing a protective action to the mucin.

Conclusions.

1. Maxwell's experiments on the inhibitory effect of colloidal starch on peptic digestion are confirmed and extended to natural gastric juice.

2. The amylase of saliva hydrolysing the starch removes its inhibitory effect on gastric digestion.

3. Pepsin and rennin behave towards colloidal starch differently—the action of the pepsin is inhibited by colloidal starch and the action of the rennin accelerated.

4. The amylase of saliva hydrolysing the starch removes its accelerating action on rennin.

5. The saliva has a delaying action of its own on milk clotting; this action was found to be due to the protective action of mucin.

6. The secretion of saliva in sucklings is important because it delays the milk clotting and thus produces finer and more easily digested curds.

I wish to express my thanks to Dr G. V. Anrep for his kind help and criticism during my experiments. The gastric juice and saliva were collected from dogs operated on by Dr Anrep.

References.

Allaria (1912). *Zentr. Biochem. Biophys.* **13**, 536.
Borisov (1914). Quoted after Babkin's *Die äussere Sekretion der Verdauungsdrüsen*, **8**, 19.
Brennemann (1917). *Arch. Pediatrics*, **34**, 81.
Maxwell (1915). *Biochem. J.* **9**, 323.

XXXI. THE RELATION OF THE FAT SOLUBLE FACTOR TO RICKETS AND GROWTH IN PIGS. II.

By JOHN GOLDING, SYLVESTER SOLOMON ZILVA, JACK CECIL DRUMMOND and KATHARINE HOPE COWARD (*Beit Memorial Research Fellow*).

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(*Received March 14th, 1922.*)

The experiments described in this communication form a part of an inquiry now in progress, the main purpose of which is to study the part played by the accessory factors in the nutrition of agricultural stock and in the etiological factors concerned in the causation of rickets in pigs. In a previous communication [Zilva, Golding, Drummond and Coward, 1921] in which the etiology of rickets in pigs was investigated we have shown that a rigorous elimination of vitamin *A* from the diet of young pigs did not conduce to the production of well-defined rickets. We were, however, able to demonstrate in those experiments that such a dietetic deficiency has a very marked effect on the development of these animals. Indications were also obtained that the deprivation of this dietetic principle possibly has some bearing on the production of healthy young.

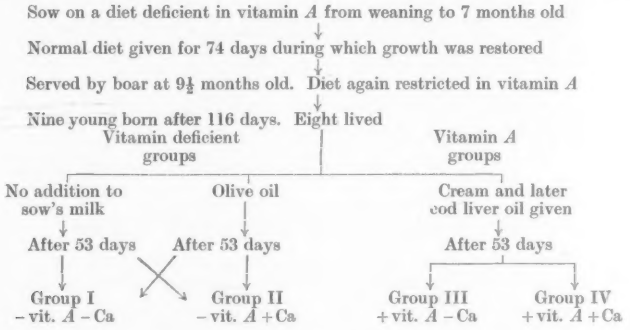
Having been unsuccessful in producing rickets in pigs experimentally by depriving them of vitamin *A* alone we next attempted to ascertain whether a dietetic deprivation of calcium and vitamin *A* would lead to the production of the disease. The results obtained by us form the subject of this communication.

Experimental.

Our animals were divided into four groups. Group I received a diet deficient in vitamin *A* and in calcium ($-A-$ Ca), group II received a diet deficient in vitamin *A* ($-A+$ Ca), group III received a diet deficient in calcium only ($+A-$ Ca), group IV received a diet containing calcium and vitamin *A* ($+A+$ Ca). Two pigs were placed in each group. Group I consisted of two boars, group II of one boar and one sow, group III of two sows and group IV of two boars. The young pigs, which belonged to the same

litter, were started on their special diets when they were 53 days old. They were young Berkshires, born of a sow 14 months old. The sow was kept for some time on a diet of toppings and whey deficient in vitamin *A* and manifested this deprivation by retarded growth [Drummond, Golding, Zilva and Coward, 1920]. On supplementing the above diet with lucerne the animal resumed growth and continued an apparently normal existence. After 74 days of correct feeding she was served by a pedigree boar and soon after was placed again on a diet of toppings, whey and swedes. This diet which was poor in vitamin *A* was purposely planned as we did not desire the young pigs to be born with a store of the vitamin. After 116 days nine pigs were born and at the age of 65 hours were divided into two sections. The chart below shows the history and subsequent allocation of the pigs. One lot received its supply of vitamin *A* entirely from the mother, the other received additional vitamin *A* in the form of cream ($\frac{1}{8}$ oz. per day) and eventually in the form of cod liver oil ($\frac{1}{2}$ rising to 1 oz. per day). Two of the animals in the former section received an equal supply of additional oil in the form of inactive olive oil, made into an artificial cream at first and later given to balance in nutrients the cod liver oil given to Section II.

The two remaining pigs in the first section received only the sow's milk; the sow being fed all the time on a diet shown to be poor in the vitamin *A*.


Sow on a diet deficient in vitamin A from weaning to 7 months old
Normal diet given for 74 days during which growth was restored
Served by boar at 9½ months old. Diet again restricted in vitamin A
Nine young born after 116 days. Eight lived
Vitamin deficient groups
Vitamin A groups
No addition to sow's milk
Olive oil
Cream and later cod liver oil given
After 53 days
After 53 days
After 53 days
Group I – vit. A – Ca
Group II – vit. A + Ca
Group III + vit. A – Ca
Group IV + vit. A + Ca

As soon as it was convenient the young pigs were given additional toppings which at the end of this preliminary period of 53 days (period I) reached a ration as high as 3 lbs. per day for the litter of eight pigs. The actual intake of food and the corresponding increase in weight for the entire experiment is summarised in Table I. The growth of the animals is graphically represented in Fig. 1. The average daily gains in pounds for period I were for pigs in section I receiving no oil 0·533 and 0·514, for those receiving olive oil 0·377 and 0·5, while the daily gains of the pigs in section II receiving cod liver oil were 0·481, 0·453, 0·344 and 0·509.

The agreement between the rates of growth of the pigs in the two sections is made more evident by employing the formula advised by R. A. Fisher,

Table I

		Group I			Group II			Group III			Group IV		
		Boar lbs.	Per 2 pigs lbs.	Boar lbs.	Sow lbs.	Per 2 pigs lbs.	Boar lbs.	Sow lbs.	Per 2 pigs lbs.	Sow lbs.	Boar lbs.	Per 2 pigs lbs.	Boar lbs.
Period I	Weight on June 3rd	29·5		30	22·5		30·5	28·5		26·5	21		30
With the sow	" April 11th	3·0		2·75	2·5		2·25	3·0		2·5	2·75		3
April 11th to	Gain in 53 days	26·5		27·25	20·0		28·25	25·5		24·0	18·25		27
June 3rd	Gain in lbs. per day	0·5		0·514	0·377		0·533	0·481		0·453	0·344		0·509
	Relative growth rate	4·31		4·50	4·14		4·91	4·25		4·45	3·83		4·34
	Oil given	Olive		None	Olive		None	Cod liver		Cod liver	Cod liver		Cod liver
		− Vitamin *A*						+ Vitamin *A*					
		Low calcium			+ calcium and phosphate			Low calcium			+ calcium and phosphate		
Period II	Weight on July 27th	50·38		51	46		56	63·125		54·56	55·625		70
Of equal intake	Gain in 54 days	20·88		21	23·5		25·5	34·625		28·06	34·625		40
June 3rd to	Relative growth rate	0·99		0·98	1·32		1·13	1·47		1·34	1·80		1·57
July 27th	Toppings, dry matter		170			170			170			170	
	Caseinogen		5·7 H			5·7 H			5·7 U			5·7 U	
	Oil		6·06 O			6·06 O			6·06 C			6·06 C	
	Chalk		—			2·0			—			2·0	
	Charcoal		4 W			5·36 A			4 W			5·36 A	
	Pounds dry matter per 1 lb. increase in live weight		4·4			3·8			3·0			2·5	
Period III	Weight on Aug. 31st	55·0		52·25*	59		71·75	84·75		75	85		99
July 27th to	Gain in 35 days	4·62		1·25	13		15·75	21·6		20·4	29·4		29
Aug. 31st	Relative growth rate	0·25	Per 1 pig	0·08	0·71		0·71	0·84		0·91	1·21		0·99
	Toppings, dry matter		55·2			111·5			155			157·28	
	Caseinogen		2·85 H			5·7 H			6·2 U			6·2 U	
	Oil		2·8 O			5·6 O			5·6 C			5·6 C	
	Charcoal		1·12 W			4·75 A			2·25 W			4·75 A	
	Chalk		—			2·2			—			2·2	
	Pounds dry matter per 1 lb. increase in live weight		14·6			4·5			4·0			3·02	
Period IV	Weight on Sept. 22nd	65·25			68·25		81·5	108·25		93·25	116·5		129·25
Unheated casein-	Gain in 22 days	10·25			9·25		9·75	23·5		18·25	31·5		30·25
ogen for all	Relative growth rate	0·78			0·66		0·58	1·11		0·99	1·43		1·22
groups	Toppings, dry matter		29·69			42·5			146			147·8	
Aug. 31st to	Caseinogen, "White Light"		1·8			3·9			4·12			4·12	
Sept. 22nd	Oil		1·52 O			3·28 O			3·52 C			3·52 C	
	Charcoal		1·75 W			3 A			2·6 W			2·3 A	
	Chalk		—			1·1			—			1·37	
	Pounds dry matter consumed per 1 lb. increase in live weight		3·39			2·83			3·74			2·57	

	Pounds dry matter consumed per 1 lb. increase in live weight		3·59		2·83			3·74			2·37			
Period V	Weight on Sept. 29th	64·75			70		82	114·25		99·5	125			1·42
Groups I and II	Gain in 7 days	− 0·5			1·75		0·5	6·0		6·25	8·5			12·75
(*a*) Heated caseinogen	Relative growth rate	Loss			0·36		0·09	0·77		0·92	1·0			1·34
	Toppings, dry matter		10·2			13·72			57·08			58·41		
	Caseinogen		0·62 H			1·3 H			1·3 U			1·3 U		
	Oil		0·52 O			1·12 O			1·12 C			1·12 C		
	Charcoal		0·44 W			0·87 A			0·87 W			0·87 A		
	Chalk		—			0·42			—			0·43		
	Pounds dry matter in food per 1 lb. increase in live weight		—			7·75			4·92			2·92		
(*b*) Unheated caseinogen	Weight on Oct. 2nd	69			71·5		86·5	116		101	130			145·5
	Gain in 3 days	4·25			1·5		4·5	1·75		1·5	5			3·5
	Relative growth rate	2·11			0·7		1·78	0·51		0·50	1·31			0·81
	Toppings, dry matter		4·64			7·96			25·32			26·55		
	Caseinogen		0·28 U			0·56 U			0·56			0·56 U		
	Oil		0·24 O			0·48 O			0·48 C			0·48 C		
	Charcoal		0·2 W			0·37 A			0·37 W			0·37 A		
	Chalk		—			0·18			—			0·18		
	Pounds dry matter per 1 lb. increase in live weight		1·26			1·59			8·23			3·31		
(*c*) Heated caseinogen	Weight on Oct. 22nd	76·25			80·75		93	135·5		120·25	162			167
	Gain in 20 days	7·25			9·25		6·5	19·5		18·25	32			21·5
	Relative growth rate	0·50			0·61		0·36	0·776		0·87	1·10			0·69
	Topping's dry matter		25·2			57			180			197		
	Caseinogen		1·95 H			3·9 H			3·9 U			3·9 U		
	Oil		—			—			—			—		
	Charcoal		1·3 W			2·6 A			2·6 W			2·6 A		
	Chalk					1·3			—			1·3		
	Pounds dry matter per 1 lb. increase in live weight		3·9			4·1			4·9			3·8		

* = Weight of Pig, group I on Aug. 25th. *Caseinogen* U = Unheated, H = Heated. *Oil* C = Cod Liver Oil, O = Inactive Olive Oil. *Charcoal* A = Animal Charcoal, W = Wood Charcoal.

NOTE. The total dry matter consumed is calculated on the ingredients given and expressed in pounds and decimals of a pound. The small daily dose of lemon juice and the occasional doses of marmite are set against loss of charcoal and chalk which were not always cleaned up.

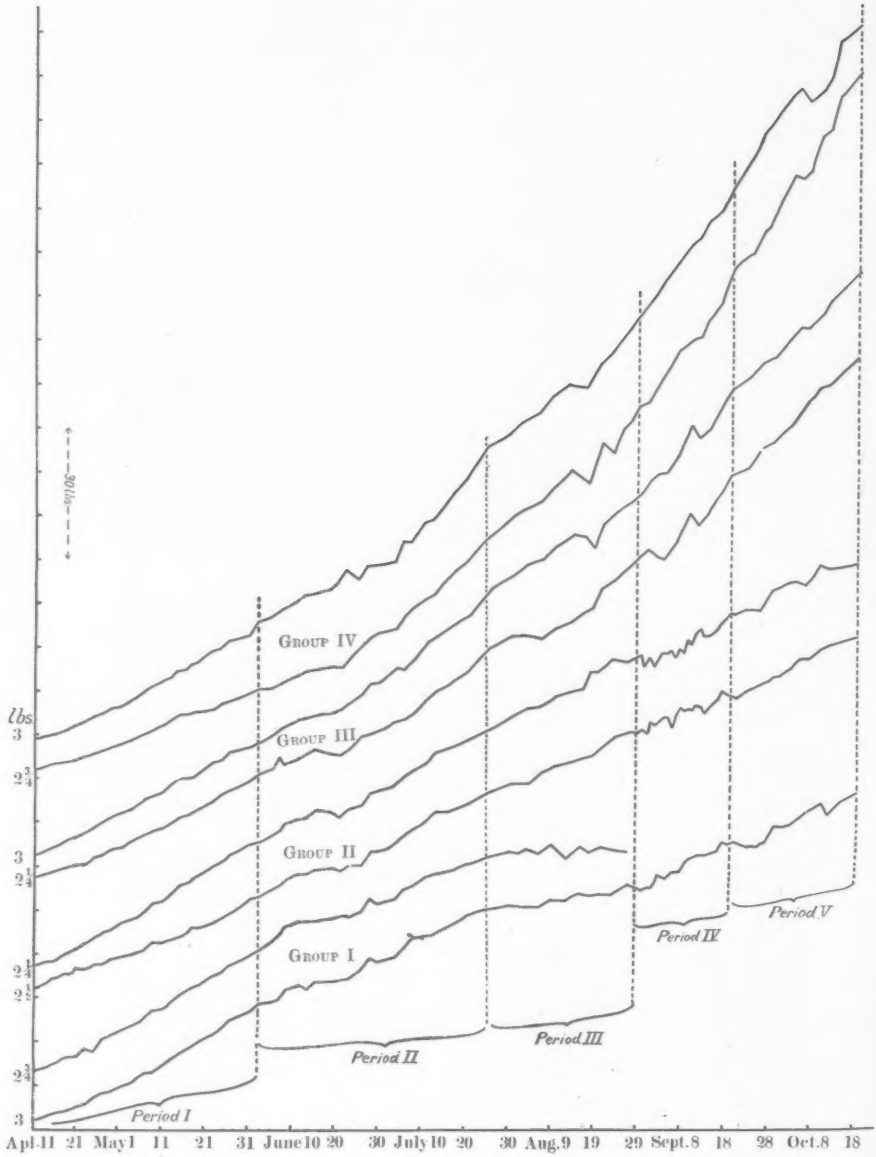

30 lbs.
Group IV
Group III
Group II
Group I
Period I
Period II
Period III
Period IV
Period V
lbs
3
2¾
3
2¼
2¼
2½
2¾
3
Apl. 11 21 May 1 11 21 31 June 10 20 30 July 10 20 30 Aug. 9 19 29 Sept. 8 18 28 Oct. 8 18

Fig. 1.

Chief Statistician, Rothamsted Experimental Station, for calculating the relative growth rate per day per cent. using natural logarithms, viz.:

$$\frac{\log_e M_2 - \log_e M_1}{K_2 - K_1} 100$$

where M_2 = the weight at the end of the period,

M_1 = „ „ commencement of the period,

$K_2 - K_1$ = duration of period in days.

The following figures are obtained for Section I ($-A$) without oil 4·91 and 4·50, with olive oil 4·14 and 4·31, for Section II ($+A$) 4·25, 4·45, 3·83 and 4·34.

It is evident from the above figures that a sow, even when fed on a diet deficient in the fat-soluble factor and having undergone a previous deprivation in this factor, is capable of rearing her young satisfactorily. This confirms further our earlier observation that the requirements of the pig for the fat-soluble factor are not of a high order.

At the end of this period, namely 53 days after birth, the animals were weaned and placed on their respective experimental diets. Each group was placed in a separate sty. The sties faced south and were divided by wooden partitions, the floor being partly wood and partly concrete. The bedding consisted of wood shavings or sawdust.

The basal diet for all groups consisted of toppings or wheat offal having the following average composition:

Moisture	11·51 %	Mucilage	59·60 %
Oil	4·29	Woody fibre	5·86
Protein	14·81	Ash	3·93

The dry matter of the above contained 0·338 % calcium. Besides the basal diet the pigs received supplementary protein in the form of caseinogen, which in the case of the vitamin-free diets was previously inactivated by being heated for 24 hours at 120° C. This inactivation was carried out for us by Dr R. T. Colgate in Messrs. Huntley and Palmer's Laboratory, for which we wish to express our thanks.

The food was weighed out three times a day at 8 a.m., 12 noon and 4.30 p.m.; it was mixed with cold water just before feeding and given in the form of a thin cream. The food was given on a live weight basis, being regulated to the quantity which the pigs would clean up.

The other two accessory factors, namely the antiscorbutic and the anti-neuritic factors, were supplied in the form of freshly prepared lemon juice and marmite (Commerical Yeast extract), about 7 cc. of the former being the daily dose, whilst $\frac{1}{8}$ oz. of the latter was given at intervals. Groups III ($+A - \mathrm{Ca}$) and IV ($+A + \mathrm{Ca}$) received a daily dose of cod liver oil as a source of the fat-soluble factor whilst the other two groups received an equivalent dose of inactive olive oil. Groups II and IV also received a daily dose of 1 oz. each of precipitated chalk and 1 oz. animal charcoal containing

67·3 per cent. of calcium phosphate as a source of additional calcium and phosphate; the other two groups received only the small amounts of calcium from the basal diet.

During the following 54 days (period II) the intake in all the four groups was the same. The increase in weight in the animals of the various groups however showed a marked disparity. The two animals in group I gained 41·88 lbs., in group II 49 lbs., in group III 62·7 lbs., and group IV 74·6 lbs. The relative growth rates, as calculated from the formula given above, were group I 0·99 and 0·98, group II 1·32 and 1·13, group III 1·47 and 1·34, and group IV 1·80 and 1·57. The average dry matter in the food consumed per pig for each pound of gain in live weight was: group I 4·4 lbs., group II 3·8 lbs., group III 3·0 lbs. and group IV, 2·5 lbs. By this time very marked differences in the general appearance of the animals in the respective groups could be discerned. The animals in group I developed a scurfy skin and saddle back, weak legs and joints painful to pressure. They were easily tired and were not playful. Those in group II also showed lack of vitality and a saddle back, those in group III were in good condition possessing glossy coat, whilst the animals in group IV were decidedly in the best condition. One animal in group I received an injury from a fall and died 87 days after the commencement of the experiment. At the post mortem examination it was found that the vertebral column was broken.

During period III, *i.e.* 35 days following period II, the increase in weight was as follows: group I 4·62 lbs. (one animal), group II 28·75 lbs., group III 42 lbs., and group IV 58·4 lbs., and the average dry matter in the food consumed per pig for each pound of gain in live weight was: group I (one animal) 14·6 lbs., group II 4·5 lbs., group III 4·0 lbs. and group IV 3·02 lbs.

Owing to the low condition of the remaining animal in group I it was decided to administer a small amount of the fat-soluble factor in order to save the pig. This was done by introducing caseinogen, which had not been previously inactivated by heating, in the diet during period IV. This addendum had its desired effect and the animal responded after about five days by resuming growth.

During this period of 22 days (period IV) the animals gained in weight as follows: group I (one animal) 10·25 lbs., group II 19 lbs., group III 41·7 lbs., and group IV 61·7 lbs. In the last period (period V) the inactivated caseinogen was alternated with crude caseinogen in order to keep the weight of the animals in group I and group II in check.

The experiment was terminated 145 days after its commencement. The animals were slaughtered with the exception of one sow in each of the groups II and III. The condition of the animals before slaughter was as follows: groups I and II wrinkled skin, ears carried forward, down on hind legs; group III skin rather rough, lack of size and bloom, flesh not quite firm, otherwise normal; group IV skin healthy and animals perfectly normal.

The photographs of the pigs before slaughtering are shown in Plate II,

two photographs having been taken of the pig in group I. The photographs were taken to scale.

The post mortem examination revealed no abnormalities of the organs beyond that the ribs of the animals in group I were easily fractured. It is also to be reported that the fat of the pigs of groups I and II was, in the butcher's opinion, softer and not of such good quality as the fat of the other pigs. This was confirmed by the estimation of the melting and solidifying points of the fats.

	Melting points		Solidifying points
Group I	21°	22·9°	19°
,, II	21°	23·8°	19·3°
,, III	25°	25·20°	22°
,, IV	31·5°	32·8°	22·5°

Table II gives the size, weight, breaking points, and calcium content of the bones.

Table II.

	Weight of humerus in g.	Distance of bearing points in inches	Breaking weight in tons	Same corrected to 3½″ length	CaO percentage of dry matter
Group I	103	3½	0·210	0·210	29·52
,, II	127	3½	0·345	0·345	29·05
,, III	117	4	0·292	0·336	33·52
,, IV	152	4	0·466	0·532	38·08

No abnormal flavour or taste was reported by a number of people who consumed the joints derived from the animals fed on the cod liver oil.

The fifth, sixth and seventh ribs of one animal in each group were examined for us histologically by Prof. V. Korenchevsky to whom we are also indebted for the interpretation of the results. The following is a summary of the observations made:

(1) Group IV (+ *A* + Ca) showed a somewhat abnormal picture with very slight osteoporosis and a belated deposition of lime salts in the newly formed bone.

(2) In all cases the bone marrow especially in the region of the secondary spongiosa consisted of a fine fibrous reticulum with but few bone marrow cells.

(3) The histological condition in animals belonging to groups II (− *A* + Ca) and III (+ *A* − Ca) was not very different from that of group IV (+ *A* + Ca). Only a higher degree of osteoporosis resulting from a diminished activity of osteoblasts could be seen.

(4) In group I (− *A* − Ca) the condition of osteoporosis was more pronounced. Moreover a more frequent incursion of the proliferating cartilage into the bone marrow was in evidence. In these places was also noticed defective calcification in the zone of provisional calcification.

It is quite evident that in spite of the very marked changes which have been effected by our restricted diets, no rickets in the pathological sense of the word has been induced. The animals in groups I and II have on various

occasions during the experiment displayed a condition which would have been described by the practical man as the pigs being "off their feet." No doubt such a condition has been before now loosely referred to as "rickets." Although defective calcification was found in the zone of provisional calcification in the case of group I ($-A-$ Ca) no increase in the amount of osteoid tissue could be established and therefore no faulty deposition of calcium in the newly formed bone in the sense of rickets can be asserted.

A remarkable feature in our experiments is that even in the case of group IV ($+A+$ Ca) which acted as our control, a normal histological picture was not obtained. This requires further investigation. Possibly the restricted diet of the mother may be responsible for this. Another point to be considered is that the animals consuming the calcium-deficient diet received 0·338 % of calcium in their food. With animals, such as pigs, which consume large bulks of food it was difficult from a technical point of view to reduce this calcium intake. However the calcium deficiency was definite enough to impair the growth and diminish the calcium content in the bones of the animals in group III ($-$ Ca $+A$) and it is very doubtful whether pigs which develop rickets under farm conditions, receive a diet much poorer in this element. We refrain from reviewing the literature which has appeared during the last few months in connection with the etiology of rickets. Most of the experimental work was done on rats and the results and conclusions of the various investigations are conspicuously contradictory. We cannot however conclude without briefly referring to the results obtained by Korenchevsky [1921]. This investigator, working with rats, obtained a definite condition of rickets on a diet free from the fat-soluble factor and calcium. Whether our apparently different results were due to the higher content of calcium in our experimental diets, or whether it was due to the different character of the experimental animal will most probably be decided by future investigation. We are continuing our experiments and although the dietetic hypothesis of the etiology of rickets forms our main line of work, we are not excluding such a hypothetical factor as light, especially in view of the latest work of Hess and Unger [1921], of Powers, Park, Shipley, McCollum and Simmonds [1922].

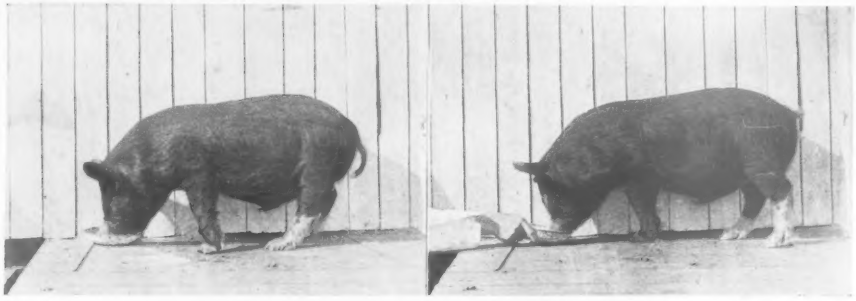
The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

REFERENCES.

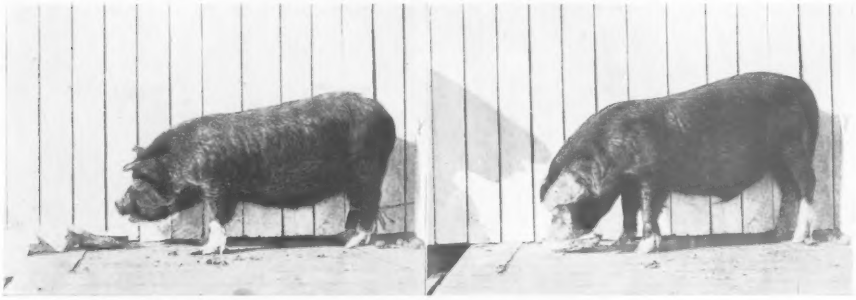
Drummond, Golding, Zilva and Coward (1920). *Biochem. J.* **14**, 742.
Hess and Unger (1921). *Soc. Exp. Biol. Med.* **18**, 298.
Korenchevsky (1921). *Brit. Med. J.* 547.
Powers, Park, Shipley, McCollum and Simmonds (1922). *J. Amer. Med. Ass.* **78**, 159.
Zilva, Golding, Drummond and Coward (1921). *Biochem. J.* **15**, 427.

PHOTOGRAPHS OF PIGS TAKEN ON OCT. 10TH, 1921

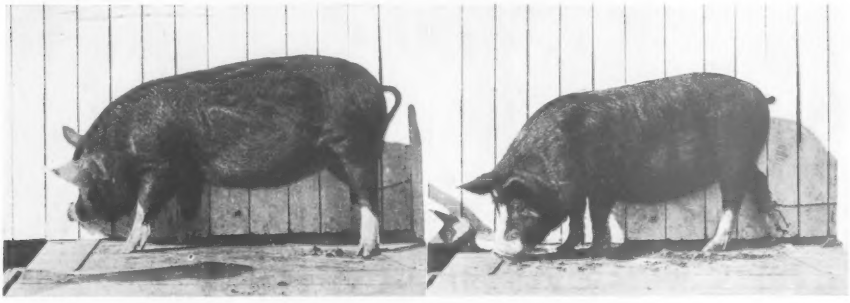
Group I – Vitamin *A*.



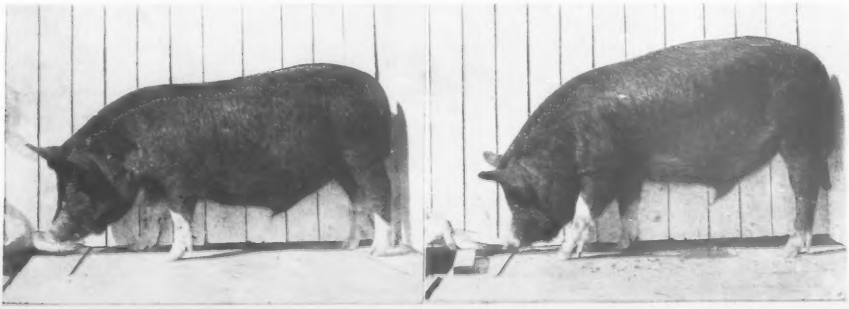
Group II – Vitamin *A* + Calcium and Phosphate.



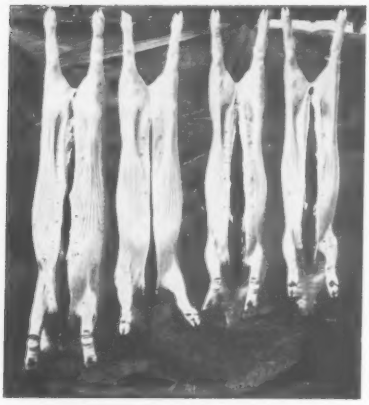
Group III + Vitamin *A*.



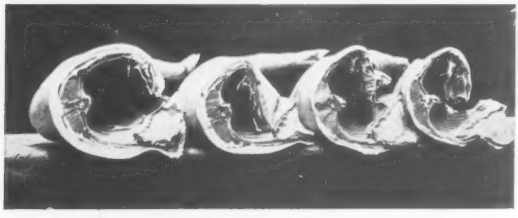
Group IV + Vitamin *A* + Calcium and Phosphate.



Pigs from Groups IV, III, II, I.



Pigs from Groups IV, III, II, I.



Cross sections of pigs from Groups IV, III, II, I.
Group I – Vitamin *A*.
Group II – Vitamin *A* + Additional Calcium and Phosphate.
Group III + Vitamin *A*.
Group IV + Vitamin *A* + Additional Calcium and Phosphate.

XXXII. THE ESTIMATION OF CALCIUM IN BLOOD[1].

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From the Department of Biochemistry of Fermentation, University of Birmingham.

(*Received March 17th, 1922.*)

Introductory.

The estimation of calcium in blood, serum etc., is a matter of extreme delicacy seeing that, in the case of human blood at least, it is not often easy to obtain more than 1 to 5 cc. of the sample. Working with such small quantities therefore it is necessary to employ a method capable of measuring calcium to a limit of accuracy of at least 0·006 mgm. It is obvious also that in these circumstances the reagents employed must be of the highest purity, especially so far as their freedom from calcium is concerned.

Among published methods are some based on the use of the haemacytometer (counting the calcium oxalate crystals) and on principles, such as (1) the determination of the concentration of calcium ions necessary to produce clotting of the blood, (2) mixing the blood with ammonium oxalate solutions made isotonic with sodium chloride, and calculating the calcium from that concentration of oxalate which just prevents clotting, (3) mixing the blood with sufficient ammonium oxalate to prevent coagulation and subsequently determining the quantity of calcium chloride necessary to produce clotting. We agree with de Waard [1919] that these give comparative rather than absolute results. Recourse must therefore be had in our opinion to chemical methods.

McCrudden [1909] suggests a gravimetric method. The calcium is precipitated as oxalate in a solution in which the hydrogen ion concentration is so adjusted that the iron and phosphates remain in solution.

Halverson and Bergeim [1917] propose to estimate calcium in blood which has not been incinerated, but from which the proteins have been precipitated by picric acid. The precipitation of the calcium is carried out under McCrudden's conditions, the hydrogen ion concentration being adjusted to the point at which alizarin gives a violet coloration. The calcium oxalate is washed by centrifuging and after dissolving it in dilute sulphuric acid the solution is titrated with $N/100$ potassium permanganate. This method, according to the examples shown in the paper, gives results agreeing closely,

[1] This investigation was undertaken at the request of my colleague, Prof. Carlier. (A. R. L.)

i.e. within the limit of experimental error, with similar estimations made on the ash of the blood, etc.

Marriott and Howland [1917] have devised an ingenious method for the estimation of calcium and magnesium in blood serum. It depends on the fact that solutions of ferric thiocyanate are decolorised by oxalates and by phosphates. Calcium is precipitated as oxalate and magnesium as ammonium magnesium phosphate. The precipitates are dissolved in acid, and added to solutions of ferric thiocyanate, the degree of decolorisation being determined by comparison in small Nessler tubes. It is said to be possible by this method to work on 2 cc. samples of serum with a maximum error of less than 5 %.

Jansen [1918] describes a gravimetric method of estimating calcium. He incinerates the blood; and he considers the removal of iron and phosphoric acid necessary before precipitating the calcium as oxalate.

De Waard [1919] has devised a method for the estimation of calcium in organic substances for which he claims an accuracy of 4 %. The substance is incinerated, the ash dissolved in hydrochloric acid in a special tube, drawn out to a capillary end, which is subsequently used for centrifuging the liquid when the calcium has been precipitated. The tube containing the hydrochloric acid solution of the ash is placed in a bath of boiling water, 0·5 cc. of saturated ammonium oxalate solution added, then a slight excess of ammonia, and finally a slight excess of acetic acid. In these circumstances he finds that the iron, magnesium and phosphates remain dissolved, whilst the calcium is precipitated quantitatively.

Experimental.

The method we have adopted is based on the methods of McCrudden, Halverson and Bergeim, and de Waard.

The blood is incinerated in a platinum dish, the ash dissolved in concentrated hydrochloric acid, and the solution washed into a centrifuge tube of special shape. In this tube the calcium is precipitated as oxalate as described later. When the liquid is centrifuged, the precipitate collects in the narrow tube leaving the supernatant liquid clear. This liquid can then be removed by a tube drawn out to a capillary without disturbing the precipitate. The calcium oxalate is washed twice in this way, dissolved in dilute sulphuric acid and the solution titrated with $N/100$ potassium permanganate.

The shape of the centrifuge tube is shown in Fig. 1. It has the following dimensions: diameter 1·5 cm., total length 12 cm., length of narrow tube 1·3 cm., bore of capillary tube 0·5 cm. The tube is graduated for 2 cc. and for 25 cc. A glass rod capable of entering the narrow tube (as shown) is used for stirring the precipitate after the addition of sulphuric acid before titrating. The advantage of this centrifuge tube is that it is of such dimensions that all the operations can be performed in it. It is large enough for

the precipitation to be carried out, the narrow tube at the base enables the precipitate to be washed easily without being disturbed, and finally the end point can be seen easily when titrating as the tube does not taper gradually to a point. The solutions may be titrated to 0·01 cc.

The procedure is as follows:

Incineration. About 2–5 cc. of blood is measured into a platinum dish. This is heated on a piece of wire gauze with a coating of asbestos, by a very small flame until all the water has evaporated and a spongy mass of carbonaceous matter is left, when it is heated more strongly. During the final heating the dish is placed on a pipe-clay triangle and brought to redness. To prevent extraneous matter from gaining access to the dish during the first part of the incineration, an inverted funnel is fixed over it by a clamp. The residue is treated with 1·5 cc. concentrated hydrochloric acid 0·5 cc. at a time—each time the solution is gently warmed and washed into the centrifuge tube. The dish is then washed three times with water. Care must be taken in the operation to keep the volume to a minimum.

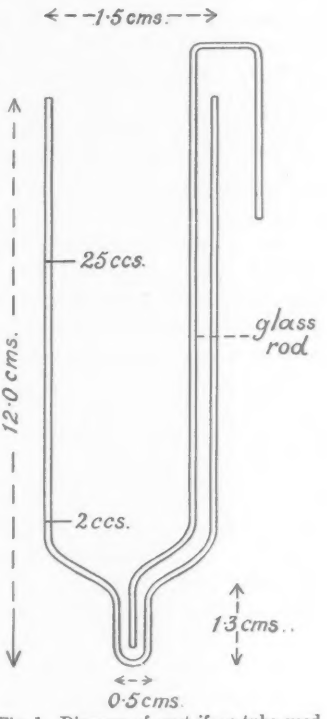

Fig. 1. Diagram of centrifuge tube used in the estimation of calcium in blood.

Precipitation. This was carried out by McCrudden's method as modified by Halverson and Bergeim. The solution in the centrifuge tube is neutralised with 0·880 ammonia using alizarin as indicator. The neutral point is overshot and then titrated back with $N/2$ HCl until the colour just commences to change. Then 2·5 cc. of $N/2$ HCl and 2·5 cc. of 2·5 % oxalic acid solution are added and water to make to the 25 cc. mark. The tube is then placed in a water-bath and brought to the boiling point. To the hot solution 5 cc. of 3 % ammonium oxalate is added and the tube kept at the boiling point for 15 minutes when it is removed, placed in ice water and 5 cc. of 20 % sodium acetate solution added (or until the colour commences to change). When the calcium oxalate has precipitated which takes about 6–8 hours (it is usually allowed to remain over night), the solution is centrifuged. The clear supernatant liquid is removed by means of a tube (drawn out to a capillary) leading to a bottle connected with a filter pump. By this means the liquid is removed to the 2 cc. mark on the tube. The outside of the capillary tube is washed with water to prevent the loss of any precipitate which may adhere to it.

Water is then added to the 25 cc. mark, the centrifuging repeated, and the supernatant liquid removed as before to the 2 cc. mark, etc. It is washed twice in this way, when 4 cc. of 5 % sulphuric acid are added and the precipitate stirred with the glass rod. The tube is then placed in a water-bath at 65° C. for 1–2 minutes, when the oxalic acid is titrated with $N/100$ potassium permanganate by means of a burette capable of reading to 0·01 cc. 1 cc. of $N/100$ potassium permanganate is equivalent to 0·020 mgm. of calcium.

A "blank" experiment must be performed on the materials. Coagulation of the blood was prevented by the addition of sodium citrate to the extent of 2·5 %.

Solutions used. All materials used were recrystallised twice from water which had been redistilled twice from a Jena glass flask, and such water was used throughout the operations. It was found necessary to have the end of the burette drawn out to a fine point and vaseline was placed on the outside so that it was possible to reduce the volume of one drop to 0·01 cc.

The permanganate solution. About 0·5 g. of potassium permanganate crystals is dissolved in 1 litre of redistilled water in a litre flask. The flask is placed on the water-bath for 2–3 days, a funnel and watch glass being placed in the neck to prevent extraneous matter from entering. The solution is then carefully filtered through an ashless filter paper on a Buchner funnel to remove the deposit of manganese dioxide. The solution should be kept in the dark. It is standardised against an oxalic acid solution prepared by dissolving about 0·25 g. of recrystallised oxalic acid in 250 cc. of water. When standardising allowance must be made for the titre of the sulphuric acid used. It is as well to standardise the permanganate about once every fortnight.

Alizarin solution. An aqueous solution of 0·2 % strength was used.

The following are some of the results obtained:

	Calcium per 100 cc. g.
1. Standard calcium solution (containing 0·0076 g. per 100 cc.)	0·0077
2. " " " " "	0·0077
3. " " " " "	0·0077
4. " " " " "	0·0077
1. Human blood	0·0082
2. " "	0·0080
1. Ox blood	0·0070
2. " "	0·0070
1. " " (clotted)	0·0055
2. " " "	0·0055
3. " " "	0·0056

In all cases 5 cc. were used for the determination with the exception of the human blood when 3 cc. and 2 cc. respectively were used.

Duplicate experiments show an agreement equal to two drops of $N/100$ permanganate or in other words to 0·006 mgm. of calcium.

REFERENCES.

Halverson and Bergeim (1917). *J. Biol. Chem.* **32**, 159.
Jansen (1918). *Zeitsch. physiol. Chem.* **101**, 176.
McCrudden (1909). *J. Biol. Chem.* **7**, 83.
Marriott and Howland (1917). *J. Biol. Chem.* **32**, 223.
De Waard (1919). *Biochem. Zeitsch.* **97**, 176.

XXXIII. THE MINIMUM NITROGEN EXPENDITURE OF MAN AND THE BIOLOGICAL VALUE OF VARIOUS PROTEINS FOR HUMAN NUTRITION.

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HISTORICAL.

Until comparatively recently, the search for the minimum protein requirements of the human body has been made on the assumption that protein is an entity, little regard being paid to whether the proteins were derived from meat, milk, cereals, etc.

The more important of the numerous investigations undertaken with this object are those of Hirschfeld [1887], Kumagawa [1889], Klemperer [1889], Peschel [1891], Lapicque and Marrette [1894], Sivén [1900] and Albu [1901][1].

In all of them the protein fed was derived from more than one source and often from several. The observations, which vary in precision, were made under conditions which were not uniform, particularly in regard to the total calories taken. Nevertheless, each experimenter succeeded in establishing that nitrogenous equilibrium could be maintained over short periods with one-third to two-thirds the standard laid down by Voit of 118 g. protein, equal to 0·39 g. N per kilo.

The minimum arrived at by the above experimenters varied between 0·08 g. and 0·18 g. N per kilo, most of the results being round about 0·1 g. The lowest value is that of Sivén, who considers that he ultimately attained nitrogenous equilibrium on a mixed diet containing 0·08 g. N per kilo, only 0·03 g. of which he regards as true protein, but as the only evidence that this small amount was sufficient is a positive balance of ·04 g. N on the last day of a four days experiment, decided negative balances occurring on the first three days, this conclusion would appear questionable. In another series in which the nitrogen intake was 37 % higher the evidence of nitrogenous equilibrium is satisfactory.

[1] The earlier observations have been collected by Atwater and Langsworthy in their "Digest of Metabolism Experiments," *Bulletin* 45, U.S. Dept. of Agriculture, 1898. An excellent review of the work previous to his paper is given by Sivén. Most of the literature on the subject to date is referred to in Mendel's "Theorien des Eiweissstoffwechsels," *Ergebnisse der Physiologie*, 11 Jahrgang, 1911; Caspari's article "Eiweissstoffwechsel" in Oppenheimer's *Handbuch der Biochemie*, 1911, and Cathcart's *Physiology of Protein Metabolism* (1921). All of these contain good bibliographies.

The investigations of Neumann [1902] and Chittenden [1904] were undertaken with a somewhat different object, namely, to ascertain whether health and activity could be maintained over prolonged periods on a mixed diet of low content in protein. Neumann made three experiments on himself, each lasting four to ten months. The total calories of the diet amounted to 30–40 per kilo. Chittenden's observations were made upon 26 individuals and the duration varied between six and nine months in different cases. The total calories varied between 35 and 45 per kilo body weight. Nitrogenous equilibrium was obtained by Neumann on an intake of 0·15 g. N per kilo and in Chittenden's experiments with 0·1 g. to 0·17 g. per kilo in the different individuals. There is no reason to suppose that these figures represent minima. The conclusion drawn is that a nitrogen intake of one- to two-thirds of the amount laid down by Voit is sufficient to maintain health and efficiency over the periods during which the observations endured.

During the last decade of the 19th century, physiologists were becoming increasingly alive to the possible significance of differences discovered in the elementary composition and chemical properties of the proteins, and the uniform value hitherto attributed to them in nutrition was coming under suspicion. Rubner [1897] appears to have been the first to formulate the view that proteins had different biological values. He used this conception to interpret some early experiments of his on the utilisation of various foodstuffs [1879] in which he had observed that less nitrogen was excreted in the urine on potato diets than on bread diets although the adverse N-balance was smaller on the former. In the same article Rubner expressed the opinion that the old search for a protein minimum must be fruitless since there will be, not one, but many minima according to the nature of the foodstuff used. He does not appear to have attributed such variation to any difference in chemical constitution although doubtless this possibility was in his mind. At this date the chemical constitution of the proteins was obscure although a number of amino acids had been isolated from the decomposition products of proteins and differences in the amounts of these had been observed. A little later Kossel and Kutscher [1900] determined the histidine, arginine, lysine and ammonia derived from the hydrolysis of a number of proteins and Kossel [1901] as a result of his own and others' work came to the conclusion that the habit of regarding protein as a physiological unit was unsound and that, since proteins possess different chemical compositions, they will also have different values for the organism.

About the same time discoveries were being made in another direction. The researches of Cohnheim [1901, 1906], Kutscher and Seemann [1901], Abderhalden and his co-workers and others, proved that a much more exhaustive break up of the protein molecule than had previously been supposed takes place in the small intestine prior to absorption, and that what the body really receives is a mixture of amino acids and simple polypeptides. Loewi [1902], and later, Abderhalden and Rona [1904] showed by means of feeding

experiments with previously digested proteins that the abiuret products of such digestion were capable of maintaining animals in nitrogen equilibrium. Abderhalden believed at first that such amino acids were at once utilised for building up of blood and tissue proteins and in conjunction with Samuely [1905] attempted to ascertain whether the composition of the body proteins varied with the character of food proteins. A horse was fed for three days on gliadin containing 36·5 % of glutamic acid, but no increase in the very low content of this amino acid in the serum proteins could be detected. From these results Abderhalden and Rona [1906] drew the conclusion that in the renewal of body proteins a proportion of the amino acids arising from the food will be left over unless the body is capable of synthesising one amino acid from another. Since this proportion will depend on the relative composition of the food and body proteins the protein minimum must also be variable.

These discoveries provided a theoretical basis for Rubner's empirical conclusions and a stimulus for the further investigation of the subject.

Meanwhile the rôle played by protein in satisfying the energy requirements of the body, and the effect on the protein minimum of insufficient as against abundant provision for these needs from non-protein sources, was becoming more clearly realised.

The discovery of the variable composition of proteins and of the fact that certain of them are almost entirely deficient in one or more of the amino acids was followed by interesting researches to determine to what extent the animal body could, by the practice of economy or synthesis, dispense with the missing complexes. The deficiency of gelatin in tyrosine was ascertained early and the absence from it of cystine and tryptophan was discovered when these amino acids became known as protein constituents. The inability of gelatin to preserve the body in nitrogenous equilibrium has been shown by many investigations, but as this aspect of the subject has recently been dealt with by one of us in this journal [Robison, 1922, 1] it is unnecessary to review it here.

The discovery of tryptophan by Hopkins and Cole [1901] and of the deficiency of this amino acid in zein by Osborne and Harris [1903] was followed by an experimental enquiry by Willcock and Hopkins [1907] to ascertain whether zein would serve as an exclusive source of nitrogen for mice and if not whether the addition of tryptophan would enhance the nutritive value of this protein. Zein alone failed to maintain the animals but this was achieved by supplementing with tryptophan.

Following this pioneer work Osborne and Mendel [1911] planned a lengthy investigation of the biological value of different proteins in the light of the new knowledge of their chemical structure. Their earlier work was carried out before the importance of accessory factors was recognised but they became aware from their experiments with individual proteins that some factor other than the supply of protein, salts and energy was complicating their observa-

tions. In the continuation of their researches, the results of which have appeared in some 30 papers in the *Journal of Biological Chemistry* from 1912 up to the present, the error due to absence of vitamins was obviated. Osborne and Mendel [1912–1920] confirmed and extended the observations of Willcock and Hopkins and showed that the addition of 3 % tryptophan to the zein given was sufficient to maintain rats over a period of 182 days, but that they failed to grow. When 2 % of lysine, in which zein is also deficient, was added, growth occurred. The problem of maintainance is therefore distinct from that of growth. The observations of Osborne and Mendel are some of the most important contributions to our knowledge of nutrition. The choice of rats enabled great numbers of experiments to be carried out and the extension of the periods of observation to cover a large fraction of the normal life of the animal. They prove that rats cannot supply some of the missing amino acids and that the minimum requirements and relative nutritive value of any particular protein depend upon the proportion of essential amino acids it contains. Their work also shows how a knowledge of the composition of particular proteins may be used for the economical adjustment of the nitrogenous portion of an animal's dietary by arranging that one protein shall compensate for the deficiencies of another.

The supplementary value of proteins from different sources has also been investigated by McCollum, Simmonds and Pitz, and McCollum, Simmonds and Parsons [1917 to 1921], whose observations, like those of Osborne and Mendel, were carried out upon rats. They found that cereal proteins could be satisfactorily supplemented by the proteins of milk, meat, kidney and casein and gelatin. Proteins of various leguminous seeds also usefully supplemented cereal proteins, *e.g.* wheat together with navy beans or peas.

The ultimate test of the nutritive adequacy of a protein is its capacity to nourish a young animal and provide for its complete growth and development and this, as far as the rat is concerned, is the criterion of the American investigators to which we have briefly referred. It may be surmised that, broadly speaking, conclusions arrived at from experiments on rats will be applicable in general to human nutrition. On the other hand the human mechanism may differ in detail. It will, for obvious reasons, be long before information as to the complete adequacy of individual proteins and quantitative data as to their biological values is forthcoming for human nutrition. In the meantime the findings of Osborne and Mendel, and McCollum and his co-workers have been applied with advantage to the feeding of stock.

From this more general survey of the subject we will now return to consider observations upon the minimum requirements for equilibrium when nitrogen is supplied in different forms. We have already referred to the observations of Rubner which led him to the conclusion that a different nitrogen minimum would be discovered for different proteins. This surmise was subsequently investigated in his laboratory by Karl Thomas [1909] who introduced the term "biological value." The expression "physiological value"

had been previously suggested by Voit and Korkunoff [1895] for a similar conception. Karl Thomas defined biological value as the number of parts of body nitrogen replaceable by 100 parts of the nitrogen of the foodstuff. Thomas's definition is not concerned with the relative digestibility of the protein. The replacement of the "Wear and Tear" quota was recognised as the only proper basis for comparison and in order to determine this value he fed himself on a carbohydrate diet (starch, sucrose, lactose) of high calorie value for periods of several days, during which the daily output of nitrogen in faeces and urine was determined. The figure to which this output fell was taken as his minimum requirements for the time being. During succeeding periods varying from one to four days a similar carbohydrate diet supplemented by a certain amount of the foodstuff under examination was taken and the N-intake and output determined as before. The N-intake was not as a rule kept constant and sometimes varied considerably on the different days. In most cases a negative N-balance was obtained. From the results Thomas calculated his biological value by three formulae based on the above definition, but differing from one another according to the way in which the nitrogen of the faeces is dealt with.

Some of Thomas's experiments lasted four to five weeks though no individual foodstuff was taken for longer than four days at a time. A period of one or two days on nitrogen-free diet was usually interposed between the experiments. Sixteen foodstuffs were investigated and their biological values recorded. These varied from 100 % in the case of milk to 30 % in the case of maize. We shall have occasion to discuss some of his results after dealing with our own experiments.

Shortly before this work of Thomas appeared the results of experiments upon dogs with a similar object were published by Michaud [1909]. The output of nitrogen on diets of dog-flesh, sugar and fat was compared with that on diets of horse-flesh, caseinogen, gliadin, and edestin and on the carbohydrate and fat alone. Nitrogenous equilibrium was attained with an amount of nitrogen in the form of dog-flesh equivalent to the nitrogen output on the nitrogen-free diet. Negative balances were obtained with the other proteins, the greatest being in the case of gliadin and edestin.

Zisterer [1910] found differences between caseinogen, flesh and gluten. These were however, in his opinion, too small to have practical significance.

Observations upon pigs were made by McCollum [1911]. These animals lend themselves to metabolism experiments of this kind as they will consume sufficient of a diet free from nitrogen to obtain the necessary calories over a considerable period. Their minimum nitrogen expenditure can therefore be determined with reasonable accuracy.

After a period of a week upon a diet of starch alone, the animals were fed for several days with the same ration to which was added a small amount of gelatin, zein, caseinogen or other protein. This was followed by the starch ration for a further period of some days. An amount of nitrogen in the

form of gelatin equal to that of the urine upon the starch diet was found to cover 39 % and in the form of zein 73 % of the animal's expenditure. The same amount of nitrogen in the form of cereal protein did not cause any rise in the nitrogen of the urine and with caseinogen the rise was small. McCollum's experiments seem to avoid all the obvious pitfalls and his results indicate a much higher biological value for cereal proteins when fed to the pig than those arrived at by Thomas's experiments upon himself.

Hindhede [1913, 2] concludes that nitrogen equilibrium may be attained on a diet of potatoes and margarine containing only 20 g. of digestible protein. The figure is, however, arrived at by deducting the nitrogen of the faeces from the intake. This method of calculation is not in accordance with our knowledge of the origin of a considerable portion of the faecal nitrogen and will furnish a too favourable balance sheet.

Hindhede [1914] vigorously contests the findings of Rubner and Thomas and claims to have attained nitrogenous equilibrium on as small an amount of protein in the form of bread as of potatoes. He declares as a result of his lengthy experiments that the proteins of potatoes, bread and meat can replace those of the body gram for gram. With Hindhede's criticisms of some of Thomas' experiments and treatment of his data, we are, for the most part, in agreement but must at the same time admit the justice of a great part of Rubner's equally severe criticisms of Hindhede's evidence, in particular, as regards the justification for assuming that all the nitrogen of the faeces represents undigested food proteins.

Abderhalden, Fodor and Röse [1915] carried out some experiments to determine the minimum requirement of nitrogen in the form of different kinds of bread and potatoes. The subject of the experiments was Hofrat Röse who possessed some peculiarly advantageous characteristics. Röse was accustomed to a monotonous diet, neither smoked nor drank alcohol and was in the habit of chewing his bolus 120 times before swallowing it. Experiments of three to eight days' duration were made on diets of potatoes, white wheaten bread, Swedish bread and kommiss brot, the last two being made from rye and containing bran. The experimental facts seem to us to warrant the conclusion that a gross intake of 4·5 g. of potato nitrogen, equal to 0·074 g. N per kilo, were adequate in the case of this Hofrat who chewed so long and so well. 9 g. N in the form of white wheaten bread was not quite sufficient and 10·8 g. N as supplied in the rye bread was only just enough to reach equilibrium. This is not, however, the interpretation placed upon the results by Abderhalden and his co-workers, who conclude that bread nitrogen is as good as potato nitrogen and that for both of them the minimum nitrogen requirement is round about 4 g. for a man of 60 kilos.

Rubner [1919] in the course of some studies of the capacity of certain vegetable nutriments to satisfy nitrogen needs, undertaken during the war, investigated different sorts of bread and the effect of milling to varying extent on the value of the product as a source of nitrogen. The paper covers a good

deal of ground and contains some particularly useful experiments with white wheat bread which can be compared with our own upon whole wheat. The bread was made, in one series, from white flour, 30 % milled, in the other of the same flour mixed with rye-bran to the extent of 30 %, so-called "Finkler brot." 10 g. of the N as contained in the fine flour and between 10 and 11 g. of that in the Finkler bread were adequate to maintain equilibrium.

Recently Sherman and his co-workers [1918, 1 and 2, 1919, 1920] have obtained results which are difficult to harmonise with those of Thomas. In experiments upon men and women, nitrogenous equilibrium was attained with an intake of 0·08 g. N per kilo, nine-tenths of this being supplied by cereal proteins and one-tenth by those of milk or apple. Wheat, maize and oats were found of equal value as a source of nitrogen and the view is taken that these cereal proteins possess a higher biological value than Thomas found. The effect of the supplementary action of the small quantity of milk may, in the light of the observations of Osborne and Mendel [1917] and of McCollum, Simmonds and Parsons [1921] be considerable.

Boruttau [1915] believes that the low value of cereals as a source of nitrogen is greatly improved when these are consumed without the removal of the bran, etc. The biological value of 145 % he obtained for the nitrogen of bran, is, in our opinion, an instance of the misuse of arithmetical formulae.

R. O. Neumann [1919] made an excellent experiment upon himself in 1917 in which he lived *exclusively* on rye bread, cane sugar and water for 40 days. Nitrogenous equilibrium was attained with 1000 g. bread and 300 g. cane sugar (= 9·9 g. N). The total calories of this diet amounted to 3630 or 63·8 per kilo. On raising the calorie value of the intake to 4434 (or 73 per kilo) the nitrogen excreted steadily fell to 7·3. This indicates that Neumann in a long continued experiment could maintain nitrogenous equilibrium on less than 7 g. of nitrogen in the form of rye-proteins if excess of calories were furnished by sugar. The experiment is also interesting as indicating the sensitiveness of the nitrogen balance to the addition of carbohydrate. This aspect of the experiment will be discussed later.

From a survey of the literature it is clear that certain of the proteins possess very different biological values both for growth and maintainance. There is, however, much uncertainty as to the degree to which the admixed proteins occurring in individual foodstuffs, where one protein to some extent complements the deficiencies of another, vary in value as a source of nitrogen.

The divergence of opinion is most marked when it is based upon metabolism experiments on man over limited periods.

OUR OWN OBSERVATIONS.

Introductory.

We commenced our investigation lightheartedly with the comparatively modest object of re-determining the relative values of certain cereal proteins in human nutrition, in particular that of maize, in view of the significance given by Goldberger and others [1915, 1920] and Wilson [1921] to the low biological value of maize in the causation of pellagra. The difficulties in arriving at values which could justifiably be compared were soon, however, apparent and it became essential to investigate thoroughly the conditions under which valid results might be obtained. In so far as the problem can be solved by metabolism experiments on adult animals the one unexceptional way to determine the relative biological values of proteins would be to ascertain the minimum intake on which nitrogen equilibrium can be maintained in each case. This sounds simple but unfortunately a positive balance only tells one that the intake is sufficient but not how much it is in excess and a number of experiments have to be performed to ascertain the minimum quantity.

We were ourselves the subjects of the experiments. This is inconvenient but advantageous, for the experiments are exacting and necessitate constant supervision of one's actions if sources of error are to be avoided. The partial abandonment of the joys of life is to some extent compensated by interest in the results.

Nevertheless, the unnecessary multiplication of irksome experiments on one's self, each extending over many days, is a thing to be avoided and it would be very desirable if a couple of observations could be made and the minimum requirements calculated from these with sufficient accuracy. This is what Thomas attempted to do. But in adopting such a method an assumption is made, the truth of which is by no means self-evident, namely, that the value of any protein for biological purposes remains uniform whatever the amount taken. The assumption would be justified if the nitrogen were utilised in the first instance to form some complex, such as "Vorratseiweiss."

In this case the biological value, as pointed out by Abderhalden, would be determined by the ratio of the percentages, in food protein and body complex respectively, of that amino acid for which this ratio has the lowest value, unless the body has the capacity to synthesise that particular amino acid from others.

It might also be true if the nitrogen requirements are of varying nature so long as they are also indivisible, that is that no single requirement can be satisfied unless at the same time all the others are satisfied.

The former of these two conceptions would appear to have been accepted without question by Rubner and Thomas though the case of gelatin obviously could not be treated in this way. Gelatin was considered to be capable of sparing body protein to the extent of 30–40 % when fed in relatively small

amounts but unable to do more than this however much was taken. Its biological value, if calculated by any of Thomas's formulae would therefore appear quite appreciable when the intake was small but almost zero if the intake was very large. Yet, Boruttau [1919] has actually made use of these formulae to calculate the biological value of gelatin and has obtained a result of 58·2 %.

Another possible disturbing factor (which we have reason to suppose occurs) is the varying economy with which the body deals with the amino acids supplied to it, according to their abundance.

The various possibilities stated above may be made clearer by a diagram in which abscissae represent real nitrogen intake and ordinates the real nitrogen output.

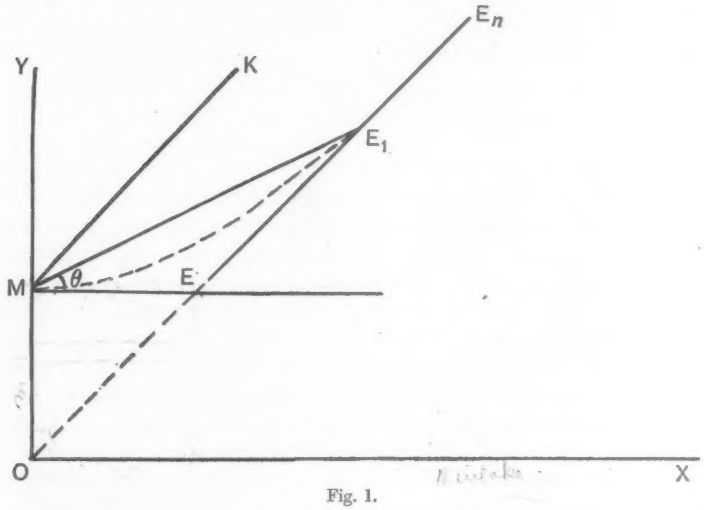

Fig. 1.

Let OM $(= m)$ be the output on a N-free diet of adequate fuel value. Then m is equal to the nitrogen minimum.

Suppose that an ideal protein (B.V. = 100) is fed in gradually increasing amounts and is utilised without waste. So long as the intake remains lower than m the output will remain constant and equal to m since the food protein saves an equal amount of body protein. The graph of intake and output will therefore be a line parallel to the x-axis and at E where $ME = MO$ the body will be in nitrogen equilibrium.

If now the intake be further increased, equilibrium will again result (unless the body is in a growing condition or has been previously starved of N) and the graph will now follow the line EE_n at an angle of 45° to the axis.

In the case of a protein of value less than that of the ideal protein just considered, equilibrium will not be attained on an intake equal to m but on

some greater amount e_1 (at the point E_1). On all amounts less than this, the output will exceed the intake and the graph will follow some line joining ME_1. Whether this line is straight or curved will depend on the conditions set out above, viz.:

(1) indivisibility of the nitrogen requirements of the body;

(2) uniform economy with varying nitrogen intake.

If these conditions are obtained the line ME_1 will be straight and its equation will be $y = m + x \tan \theta$ where y is the real output corresponding with any real intake x less than e_1.

For higher values of x the graph will follow the line E_1E_n.

Thomas's formulae can be very simply expressed in terms of θ; thus formula B

$$\text{B.V.} = 100 \frac{\text{Urine N in N-free diet} + \text{faeces N} + \text{balance}}{\text{N-intake}}$$

becomes

$$\text{B.V.} = 100 \frac{m + (x - y)}{x}$$

$$= 100 \frac{m + x - (m + x \tan \theta)}{x}$$

$$= 100 (1 - \tan \theta).$$

If the above conditions do not obtain, *e.g.* if a number of different amino acids are required for specific purposes, which are distinct and can be separately satisfied, the graph of a protein rich in certain of these acids but poor in others would be a curved line such as the dotted line joining M and E_1 in the diagram. This curvature would express the fact that a certain fraction of the body's needs could be satisfied by a smaller amount of this protein than would correspond with the amount required to obtain equilibrium. The angle θ and the biological value would then vary for different values of x.

The graph of a protein, unable by itself to satisfy any portion of the body's nitrogen requirements, would be a straight line MK parallel to OE_n, since the nitrogen output would always be equal to the intake + m. For this line $\theta = 45°$ and the equation $y = m + x \tan \theta$ becomes $y = m + x$ while the biological value $= 100 (1 - \tan 45°) = 0$.

In our opinion there was very little reason for assuming that these graphs would necessarily prove to be straight lines. It is true that Thomas calculates his values from individual daily balances and takes the average of the results, but such daily balances are too variable and are subject to too great experimental errors to offer any satisfactory proof of the uniformity of the value. We therefore set out to obtain evidence on this question by determining as accurately as possible a number of points for the same protein but for different values of x. Our results will be considered later but we may here state that in the case of bread proteins the points do lie on, or close to, a straight line. In the experiments with nitrogen in the form of milk results were at first obtained indicating pronounced curvature of the line, and nitrogen equilibrium was not obtained until more than 11 g. of milk nitrogen was taken per day. By increasing the amount of carbohydrate however, so that the

fuel value of the diet was greatly in excess of requirements and the respiratory quotient greater than 1, equilibrium was finally reached with half this amount, and bearing in mind that when x, the intake, is very small, the physiological errors of experiment become relatively great, the observations could now perhaps be expressed by a straight line. As long as any doubt exists of the rectilinear character of the line *ME* it will obviously be prudent to place reliance only upon observations in which x and y are as large as possible short of equality.

Factors which must be considered if valid results are to be obtained.

1. *The time required to reach a uniform N-output on a constant intake.*

The effect of the previous diet upon the N-output and the length of time required to reach a constant output on a constant intake which is either greater or less than that of the preceding period, was clearly demonstrated by the old experiments of C. Voit [1866, 1867]. The N-output of a dog during the first days of starvation varied with the amount of protein in the previous diet but fell gradually until a relatively constant figure was reached on the fifth or sixth day. When the dog was given a constant meat diet for some days and then a considerably greater (or less) amount daily during a further period a similar gradual increase (or decrease) in the N-output was observed during five or six days before equilibrium again set in at the new level. These observations have been repeatedly confirmed by Grubner [1901], Landergren [1903], Kinberg [1911] and many others.

The rapidity with which the nitrogen excretion diminishes obviously depends on the difference between the N-intake during the experimental and the preceding periods and will be greatest when a period of nitrogen starvation follows one of high protein intake or *vice versa*. There is no reason to suppose, however, that this gradual change is ever replaced by an immediate jump to the new level even when the difference between the two planes of N-intake is but small, though naturally the absolute amounts of the variations will be correspondingly less.

Whether the N-output is also influenced by the nature as well as the amount of the protein taken in the foregoing period is more difficult to decide. If part of the nitrogen of the previous diet is stored up in any form that can be utilised by the body (*e.g.* amino acids) and not merely in the form of unexcreted end products, we should expect the N-output during the first few days of the succeeding period to be influenced thereby—unless during both periods the body is in N-equilibrium. For example if the diet during the first period contains 10 g. of N from caseinogen, and during the second period a negative balance occurs on 10 g. of N from zein, the amount of this negative balance might very well be less during the first few days of the zein diet than on the latter days of the same period owing to the supplementary action of amino acids stored up during the caseinogen diet. The results of experiments [Robison, 1922] in which a diet containing gelatin as the sole protein followed

a diet of mixed proteins suggest that this does occur, and that therefore when the diet is changed in any way—either in amount or nature of the protein—the N-output cannot be considered to represent that of the second diet until some days have elapsed.

It follows that metabolism experiments are subject to error from these causes and that the error diminishes as the duration of the experiment increases. As a compromise, we have exlcuded from calculation the figures for the first three or four days in arriving at the N-output. It is also advisable that the N-intake should not vary during the experiment. Most of Thomas's experiments are subject to both these sources of error.

2. *The time required for the elimination of errors due to the fluctuation in the N-output.*

The very considerable fluctuations that may occur in the amount of nitrogen excreted in the urine by men receiving an absolutely constant diet have been noted by Bornstein [1898], Atwater and Benedict [1902] and by Falta [1906], by all of whom the cause was considered to be psychical. Atwater and Benedict also noted the increased diuresis which often accompanied a high N-output and thought it probable that the diuresis was the direct result of the psychical stimulus and the cause of the increased N-elimination. Falta observed variations of 4–5 g. in the N-output on individual days although in equilibrium over the period as a whole.

Neumann [1899] studied the influence of variations of the urinary flow upon the daily output of nitrogen, the intake remaining constant. When diuresis was produced by increasing the water drunk from one to three litres, the N-output increased from 10·5 to 14·3 g. and did not reach the original level until the third day.

In almost all our experiments such fluctuations, amounting frequently to 25 % of the mean output, have occurred and not least in those experiments in which the most rigorous attention has been paid to constancy of diet and fluid intake, and to regularity in the mode of life.

At first we also were disposed to attribute these fluctuations to increased diuresis but the frequent lack of any correlation between the two compelled us to modify our opinion. Some factors (*e.g.* mental strain or excitement) may possibly affect both diuresis and N-output, but the latter does not always coincide with the increased volume of urine and may even vary in the opposite sense. In one experiment, for example, the minimum N-output corresponded with the maximum volume of urine.

Judging from the experiments of Voit and other workers on dogs and of McCollum on pigs, it would appear that in the case of these animals the fluctuations in N-output on a constant diet are usually less considerable than with man. It is clear that calculations based upon the nitrogen balance sheet for single days, a procedure frequently resorted to, are subject to very large errors and that these can only be eliminated by taking the average results over a number of days.

3. *The necessity for abundant energy supply.*

It has been universally recognised that proteins will be used as fuel unless an adequate supply of fat and carbohydrate is provided, but there have been different opinions as to what constitutes adequacy in this respect. In their search for the protein minimum some of the earlier workers considered it necessary to supply a diet of fuel value very greatly in excess of the energy requirements of the organism. Thus in Klemperer's [1889] experiments on two young men, a diet of 5020 calories, equal to about 75 calories per kilo was given. Sivén, however, was of the opinion that such excess was unnecessary and that the minimum could be reached without increasing the calorie value of the diet above the normal. The fuel value of Sivén's diets was equal to about 40 calories per kilo body weight.

Hindhede's [1913, 2] attitude is somewhat difficult to understand. He considers that an abundant calorie intake is necessary if the protein minimum is to be attained, but that this minimum will vary with the calorie value of the diet. He does not believe that a quiet old man, for whom a diet of 1500–2000 calories is sufficient, can have the same minimum as an active young man who requires a diet of 3000–5000 calories. From the results of his experiments, he calculates by simple proportion, the minimum for a standard diet of 3000 calories. As on a diet of 3900 calories F. Madsen's minimum was equal to 25 g. of digestible protein so, according to Hindhede, for 3000 calories the minimum would be 19 g. of digestible protein.

Rubner [1919] has criticised this procedure, and in our opinion justly, on the ground that it is unwarranted by the facts, and considers that the values so calculated to 3000 calories possess no scientific basis.

Rubner's own conclusions are that the N-minimum may sometimes be reached when no more than a third of the total energy requirements are satisfied, in other cases only when they are fully met, while in others a diet considerably in excess of these requirements will be necessary. These differences he considers are due to the varying nutritional condition of the body cells. The minimum is, however, most easily reached on an abundant carbohydrate diet.

In our own experiments on a diet nearly N-free we appeared to reach our N-minimum with an intake of 45–50 calories per kilo body weight of which about one-third was taken in the form of fat, whereas on a diet of milk (with additional carbohydrate), equilibrium was not readily obtained until the fuel value was increased to about 55 calories per kilo of which only 10 % was in the form of fat.

The effect of diets containing varying proportions of fat and carbohydrate on the protein minimum has been studied by Zeller [1914], who found that the mixed diet was just as efficacious in reducing the consumption of body protein as one of carbohydrate alone, so long as the proportion of carbohydrate to fat did not fall below 1 : 4.

Neumann's [1919] experiment upon himself, with a diet composed of bread and sugar, affords a striking demonstration of the effect of excess of carbohydrate in lowering the protein minimum. It is obvious that Prof. Neumann readily stores fat. Otherwise, he could not consume 73 calories per kilo over a period of three weeks, unless doing hard work. In his case, presumably, a greater excess of carbohydrate would be required to maintain the blood sugar at a high level than in our own, owing to the greater greed of his connective tissue cells.

It would seem, therefore, that the most certain way of determining the protein minimum would be to take a diet consisting mainly of carbohydrate and so much in excess of the energy requirements that the blood sugar is maintained high, the liver and muscles are kept well stocked with glycogen and the surplus is being stored as fat, as indicated by a respiratory quotient above unity. This was accomplished in the latter part of our experiment on milk. It is by no means easy for one of the meagre habit of the subject of the experiment (C.J.M.) as it increases the distaste for the sufficiently unappetising ration of starch and lactose and if persisted in too enthusiastically it produces unpleasant symptoms.

4. *Reduction of the nitrogen in the basal diet to a minimum.*

The carbohydrate (starch, lactose, etc.) and fat, etc. which form the basal diet for these experiments are nominally but not absolutely nitrogen-free. The amount of nitrogen taken in this form can of course be estimated but its biological value is unknown and this complicates the results. It is therefore important to reduce the nitrogen in the basal diet to a minimum by careful selection of the most suitable forms of such foods.

Most observers have neglected to take account of the nitrogen in the starch, etc. fed. As large quantities of such basal ration are consumed it may not be negligible in the case of experiments in which small quantities of some protein are being given.

5. *Accessory food factors and inorganic salts.*

The duration of metabolism experiments is limited by the difficulty of providing an adequate supply of the accessory food factors. The ill effects of long continued low-protein diets observed in some of the older animal experiments was no doubt sometimes due to the deficiency of one or other of these factors.

Fat soluble *A* can be introduced in the form of rendered butter fat or cod liver oil, and water soluble *C* in the form of lemon juice, the nitrogen content of which is very low, but we have found no method of introducing the water soluble *B* without an undue amount of possibly very valuable nitrogen.

When the diet is deficient in inorganic salts or is such as to afford an acid ash, adjustment by suitable amounts of a salt mixture is essential. McCollum and Hoagland [1913] found that the endogenous metabolism of the pig reached its lowest level when the animal was given an abundant carbohydrate diet together with a salt mixture of an alkaline character. When an acid salt mixture was given the urinary output rose, the increase occurring in the amount of ammonia. They concluded that this animal is not able to use the nitrogen of the urea fraction for the neutralisation of acid.

6. *The apportioning of the nitrogen in the faeces.*

The difficulty of correctly apportioning the nitrogen in the faeces to unabsorbed food nitrogen and excretion from the alimentary tract respectively, is the limiting factor in most experiments in which the total intake of nitrogen is small, and the way in which different observers treat the faecal nitrogen has given rise to much controversy and recrimination.

The daily nitrogen output in the faeces on a protein-free diet usually amounts to about 1 g. On other diets the amount may be considerably greater than this and the question arises—what is the significance of this excess? Does it represent unabsorbed food residues or increased loss of body nitrogen? This difficulty was recognised and discussed by Karl Thomas, whose three formulae for the calculation of the biological value of protein differ only in the assumptions that are made with regard to this point. In formula A the whole of the nitrogen of the faeces is assumed to represent unabsorbed food; in formula B it is assumed to arise entirely from the body, while in formula C 1 g. N (which is taken as the average output on protein-free diet) is assumed to be body nitrogen, any excess over this amount being ascribed to unabsorbed food.

Rubner [1915] has investigated this problem in connection with his researches on the digestibility of various foodstuffs. He has devised a method for estimating the amount of nitrogen in the faeces present in the form of undigested food residues (vegetable cell membranes) by making use of the insolubility of the latter in acid alcohol and in a concentrated solution of chloral hydrate in which he states bacteria, epithelial cells, etc. are dissolved. He considers that the rest of the nitrogen comes from the body and represents metabolic products, and he concludes that such body nitrogen forms a very considerable proportion of the increase in the total nitrogen of the faeces that commonly occurs when the diet consists largely of whole cereals, vegetables or fruit.

Rubner found that on a vegetable diet nearly half the total nitrogen of the faeces was soluble in acid alcohol whilst on a diet consisting chiefly of animal foodstuffs the proportion of soluble nitrogen was still greater. We have obtained similar results with faeces resulting from a mixed diet, but do not

know in what form the whole of this soluble nitrogen is present, and are therefore not able to draw definite conclusions as to its origin.

So long as this uncertainty remains, the nitrogen of the faeces will be the limiting factor for the accuracy of such experiments as those here described. We have therefore followed Thomas's plan and have calculated our results in two ways; the first, assuming that the whole of the nitrogen of the faeces comes from the body and that this amount plus the urine N on N-free diet represents the body's minimum requirements under the conditions of the experiment; the second, assuming that the amount by which the nitrogen of the faeces exceeds the average amount excreted on a N-free diet represents unabsorbed food and is therefore to be subtracted from the total intake in order to arrive at the true nett intake, *i.e.* the absorbed nitrogen. The truth will probably lie somewhere between these two extremes.

Experimental.

Firstly as to general procedure. The N of all foodstuffs and beverages used was determined. The food was weighed in the same condition as that in which the N was determined and the total intake of N recorded in the tables can be relied upon to plus or minus 0·01 g. The urinary excretion of each 24 hours was collected in the presence of toluene, weighed, and duplicate determinations of the N content made. Care was exercised to see that invalid sampling from the deposition of either urates or ammonium magnesium phosphate did not occur. The faeces, after collection each day, were mixed with some H_2SO_4 to prevent decomposition and possible loss of NH_3 and great care was taken at the end of each period to ensure the validity of the samples taken for analysis. N determinations were made upon about 10 g. in duplicate, only closely concordant results being accepted.

The evacuation of the intestines is not usually so regular and complete that any great value can be placed on the figures for the nitrogen excretion on individual days. The best that can be done is to determine the nitrogen in the faeces for the whole experimental period and from this to calculate the average daily output. The latter may vary within rather wide limits even when the diet contains little or no nitrogen, and appears to depend to some extent on the bulk and character of the faeces. In order that these might be kept as uniform as possible agar-agar was taken when the diet consisted wholly or largely of completely digestible foodstuffs.

Food was taken in approximately equal amounts three times a day at the customary hours, and we led our usual life. In the experiments upon milk we abandoned all attempts to make our basal ration of fat and carbohydrate resemble a repast and drank a suspension of uncooked corn-starch in a saturated solution of lactose. This was followed by an alkaline salt mixture and 2 g. of agar-agar. In this way 600 calories were contained in about a tumbler full. Microscopical examination of the faeces showed that the

starch was completely digested. When the starch-lactose mixture exceeded 250 g. at one meal some glycosuria occurred temporarily. On the N-minimum experiment the faeces were olive green as the bile pigment was unreduced. Little gas was formed.

The following are the essential data regarding the subjects of the experiments, ourselves:

C.J.M. Age 56. Weight 61 kilo. Height 183 c. Very thin, stores fat with difficulty. Mode of life: laboratory work. Exercise: lawn tennis before breakfast for three-quarters of an hour and about three miles walk during the day.

R.R. Age 37. Weight 59 kilo. Height 173·5 c. Spare, does not store fat readily. Usual mode of life consists chiefly in laboratory work. Very little regular exercise beyond daily walk of two to four miles.

Minimum nitrogen expenditure on carbohydrate-fat diet.

Our minimum nitrogen expenditure was determined in two experiments, during which our diet was as nearly as possible nitrogen-free. In the vain attempt to make this diet appetising much labour was expended in endeavouring to prepare the food in a varied and attractive manner. Biscuits made of corn starch with 20 % of fat proved quite palatable when taken in small quantities but nauseating in bulk. A biscuit, whose chief defect was its hardness, made from starch, dextrin and a little fat was finally adopted and formed, with butter and honey, the chief article of diet. A starch mould flavoured with lemon juice was also taken. Weak tea with lemon and sometimes black coffee and a little vermouth was drunk. After the first few days of this diet no desire was felt either for this or for any other food, nor did the sight of our first normal meal at the close of the experiment arouse any appetite. The drinking of a glass of hot milk, however, excited in a few minutes a very keen appetite and desire for food. The quantities of the individual constituents of the diet varied somewhat from day to day but were always accurately measured and noted. Those for a single typical day are set out in Table I while the total nitrogen intake and fuel value of the diet for each day are shown in Table II in which are also set out the daily output of nitrogen in the urine and faeces and the nitrogen balance. In this and in all other experiments the nitrogen in the tea and coffee has been assumed to consist chiefly of caffeine and to be excreted unchanged in the urine. It has therefore been subtracted in all cases from the total intake and from the urinary nitrogen output. Any error involved in this method of treatment must be of negligible dimensions.

The nitrogen in the urine fell steadily until the last day of the experiment when a rather considerable rise occurred in the case of both C.J.M. and R.R. This is probably to be explained by the fact that, owing to the difficulty of consuming the food when all appetite was in abeyance, the fuel value of the diet was reduced during the last day or two.

Table I.

Foodstuff	N-minimum 2. N per 100 g.	Calories per 100 g.	Diet R.R. 4. xii. 20 Weight g.	N g.	Calories
Corn starch	0·042	360	280	0·118	1008
Dextrin	0·065	360	50	0·033	180
Butter	0·080	775	90	0·072	698
Margarine (rendered)	0·010	900	15	0·002	135
Honey	0·023	327	55	0·013	180
Sucrose	—	395	25	—	99
Lactose	0·013	370	65	0·008	241
Lemon juice	0·067	40	30 cc.	0·020	12
Vermouth	0·005	140	25 „	0·001	35
Tea infusion	0·006	—	1200 „	0·072	—
Agar-agar	0·242	—	15 g.	0·036	—
Salt mixture	—	—	5 „	—	—
			Total	0·375	2588
			Excluding tea N	0·303	45 per kilo

Table II.

Date 1920	Body weight k	Daily Intake: N (excluding tea and coffee) g.	Daily Intake: N tea and coffee g.	Daily Intake: Calories per kilo	Output: N urine g.	Output: N faeces g.	Output: N total g.	Balance N g.
Nov. 15	58·5	0·30	0·16	56	8·64	—	—	—
„ 16		0·28	0·06	54	5·31	1·05	6·36	−6·08
„ 17		0·30	0·15	55	3·63	„	4·68	−4·38
„ 18		0·25	0·10	51	2·66	„	3·71	−3·46
„ 19		0·22	0·06	43	2·25	„	3·30	−3·08
„ 20	57·7 (21. xi)	0·23	0·15	49	2·82	„	3·87	−3·64
N-minimum 2. Subject: R.R. Fluid Intake: 2000–2500 cc.								
„ 29	58·6	0·27	0·07	49	8·79	1·13	9·92	−9·65
„ 30	(23. xi)	0·30	0·07	50	4·71	„	5·84	−5·54
Dec. 1		0·30	0·08	51	3·46	„	4·59	−4·29
„ 2		0·35	0·08	51	2·71	„	3·84	−3·49
„ 3		0·32	0·09	52	1·99	„	3·12	−2·80
„ 4		0·30	0·07	45	2·17	„	3·30	−3·00
„ 5	57·7	0·27	0·06	44	2·01	„	3·14	−2·87
Average of last 3 days		0·30	0·07	—	2·06	1·13	3·19	−2·89
N-minimum 1. Subject: C.J.M. Fluid Intake: 2000 cc.								
Nov. 14	61·7	0·33	0·20	56	8·61	—	—	—
„ 15		0·30	0·20	47	4·89	1·24	6·13	−5·83
„ 16		0·30	0·20	47	3·28	„	4·52	−4·22
„ 17		0·38	0·21	52	3·28	„	4·52	−4·14
„ 18		0·32	0·21	46	2·48	„	3·72	−3·40
„ 19		0·30	0·21	45	2·25	„	3·49	−3·19
„ 20	60·9 (21. xi)	0·21	0·18	35	2·49	„	3·73	−3·52
N-minimum 2. Subject: C.J.M. Fluid Intake: 4000 cc.								
„ 29	61·9	0·34	0·31	59	8·89	—	—	—
„ 30		0·34	0·27	55	4·78	—	—	—
Dec. 1		0·34	0·33	55	3·40	1·17	4·57	−4·23
„ 2		0·33	0·32	53	2·41	„	3·58	−3·25
„ 3		0·35	0·32	52	2·51	„	3·68	−3·33
„ 4		0·35	0·29	52	2·40	„	3·57	−3·22
„ 5	60·5	0·33	0·26	48	2·13	„	3·30	−2·97
Average of last 3 days		0·34	0·29	—	2·34	1·17	3·51	−3·17

On plotting the amounts of urine nitrogen as ordinates against the time in days as abscissae it became apparent that all the points except the last would lie on, or close to a curve for which a simple logarithmic expression was found (Figs. 2 and 3). Among several possible interpretations of this curve is the simple one that the falling nitrogen output represents washing out of some metabolic products from the tissues, the amount washed out each day being proportional to that still present.

A second similar experiment was therefore carried out in order to confirm this result and also to discover whether the steepness of the curve could be altered by drinking large quantities of water and thus greatly increasing the volume of the urine. Apart from the volume of total fluid, which was doubled, the diet did not differ from that taken in the first experiment. The results are set out in Table II.

The regularity in the fall of the nitrogen output was again observed but the change in the rate of this fall was very small.

The average outputs in urine and faeces on the last three days of this experiment have been taken as representing the minimum nitrogen expenditure on such a diet. Whether this amount also represents the minimum expenditure on a diet that is absolutely nitrogen-free will depend on the biological value of the small amount of nitrogen in the food consumed during the above experiment. If this has a value of 100 %, *i.e.* if it can replace and therefore spare an equal amount of body nitrogen, the output thus determined will be equal to the real minimum expenditure. If the value of the food nitrogen is zero, *i.e.* if it is unable to satisfy any fraction of the body's requirements, it must be excreted in addition to the amount representing the latter and the minimum expenditure will therefore be equal to the observed output less the full amount of the nitrogen intake. Probably this nitrogen, which was present chiefly in the corn starch and butter, has a value intermediate between 100 and zero. The minimum nitrogen expenditure will therefore be some amount between those shown in the last two columns below.

Minimum nitrogen expenditure.

Subject	Urine g.	Faeces g.	Total output g.	Total output – intake g.
C.J.M.	2·34	1·17	3·51	3·17
R.R.	2·06	1·13	3·19	2·89

The Biological Value of the Proteins of Whole Wheat.

A series of experiments was carried out with whole wheat flour with two objects in view:

(1) to determine the Biological Value of the wheat proteins from the minimum amount with which nitrogen equilibrium can be attained;

(2) to discover whether the Biological Value is uniform for varying amounts of wheat nitrogen.

The first of these has been investigated upon man by other workers, whose

results will be considered with our own. The great practical importance of this question and the astonishing divergence between the results of previous investigations were sufficient reasons for further study.

Method of experiment.

The large variations in the percentage of water, and consequently of nitrogen, in different parts of a loaf of bread and the difficulty of obtaining a satisfactory sample, render it impossible to estimate the total nitrogen content of the loaf with sufficient accuracy for these experiments. We decided therefore, to base our calculations on the flour and to bake the bread ourselves. By suitable manipulation it was found possible to prepare loaves from 500 g. of flour with a maximum loss of less than 0·1 %. The other materials used were butter (5 %), salt, baking powder (prepared from tartaric acid, sodium bicarbonate and corn starch) and water. The loaves were baked in the laboratory for about one hour at a temperature of 240°–250°, and were only very slightly browned so that no appreciable loss of nitrogen can have occurred during the baking. For the first period, a somewhat coarsely ground whole wheat flour containing 1·85 % N was used but on increasing the daily ration from 300 g. to 450 g., considerable discomfort was experienced from the large particles of bran, and the bread was poorly absorbed. For the second and remaining periods a very finely ground flour prepared from a mixture of English and foreign whole wheat was employed. We found it very palatable and well absorbed, as the figures for the nitrogen in the faeces indicate. Only when the daily consumption had been raised to 550 g. and the total calories to 63 per kilo did we experience any discomfort.

The experiment was carried out in duplicate on ourselves and commenced with a total nitrogen intake of nearly twice the amount of our minimum requirements. On this diet a considerable negative balance occurred and the amount of wheat nitrogen was therefore increased during successive periods until equilibrium was finally attained.

The daily ration of flour was kept constant during each separate period of the experiment, but some latitude was permitted in the amounts of the remaining constituents of the diet. The actual quantities taken were, however, measured and the variations in any one period were not such as to affect appreciably the total nitrogen intake or greatly alter the fuel value of the diet. The diet set out in Table III shows the average amounts consumed by R.R. from the 24th to the 31st of October but except for the quantity of flour it would with slight variations serve for the whole experiment.

The experimental results are set out in Tables IV and V.

As in the previous experiments it has been assumed that the nitrogen consumed in tea and coffee would be excreted unchanged and the amount has therefore been subtracted from both intake and output (urine N).

During certain periods indicated by the letter (A) in the first column of

Table III.

Foodstuff	Whole wheat. N per 100 g.	Whole wheat. Calories per 100 g.	R.R. Diet during period 5, 24–31 Oct. 1920. Weight g.	N g.	Calories
Flour, whole wheat	2·162	360	450	9·730	1620
Corn starch	0·042	360	36	0·015	130
Butter	0·080	775	56	0·045	434
Dripping	0·016	885	80	0·013	708
Honey	0·023	327	40	0·009	131
Marmalade	0·052	341	20	0·010	68
Cane sugar	—	395	56	—	221
Lemon juice	0·067	40	20 cc.	0·013	8
Tea	0·006	—	960 „	0·058	—
Coffee	0·041	—	100 „	0·041	—
Vermouth	0·005	140	25 „	0·001	35
			Total	9·935	3355
			Excluding tea and coffee	N 9·836	58 per kilo

Table IV.

Diet: whole wheat. Subject: C.J.M.

Period	Date 1920	Body weight k	Daily Intake: N bread g.	Daily Intake: N total g.	Daily Intake: Calories per kilo	Daily Output: N urine g.	Daily Output: N faeces g.	Daily Output: N total g.	Balance N g.
1. (A)	Sept. 30–Oct. 2	61·80 (26. ix)	5·55	5·81	46	—	—	—	—
(O)	Oct. 3–5	61·50 (3. x)	„	5·70	44	5·62	2·14	7·76	−2·06
2. (A)	„ 10–14	60·45	8·32	8·63	56	—	—	—	—
	„ 15	(10. x)	„	„	„	8·26	2·17	10·43	−1·80
	„ 16		„	„	„	7·62	„	9·79	−1·16
	„ 17	61·44	„	„	„	6·98	„	9·15	−0·52
		Average	8·32	8·63	56	7·62	2·17	9·79	−1·16
3. (A)	„ 18		8·65	8·97	57	7·86	2·35	10·21	−1·24
	„ 19		„	„	„	7·25	„	9·60	−0·63
	„ 20		„	„	„	7·15	„	9·50	−0·53
		Average	8·65	8·97	57	7·42	2·35	9·77	−0·80
4. (A)	„ 21	60·65	9·73	9·98	53	7·25	1·74	8·99	+0·99
	„ 22		„	„	„	7·92	„	9·66	+0·32
	„ 23	61·03	„	„	„	7·30	„	9·04	+0·94
		Average	9·73	9·98	53	7·49	1·74	9·23	+0·75
5. (O)	„ 24	60·95	9·73	9·85	60	8·65	1·94	10·59	−0·74
	„ 25		„	„	„	9·23	„	11·17	−1·32
	„ 26		„	„	„	8·77	„	10·71	−0·86
	„ 27		„	„	„	7·84	„	9·78	+0·07
	„ 28		„	„	„	8·93	„	10·87	−1·02
	„ 29		„	„	„	8·63	„	10·57	−0·73
	„ 30		„	„	„	7·68	„	9·62	+0·23
	„ 31	61·60	„	„	„	7·67	„	9·61	+0·24
		Average	9·73	9·85	60	8·43	1·94	10·36	−0·51
6. (O)	Nov. 1		11·89	12·00	61	9·13	2·53	11·66	+0·34
	„ 2		„	„	„	8·33	„	10·86	+1·14
	„ 3		„	„	„	9·83	„	12·36	−0·36
	„ 4	61·40 (5. xi)	„	„	„	9·63	„	12·16	−0·16
		Average	11·89	12·00	61	9·23	2·53	11·76	+0·2

the table, a quantity of stewed apples (225 g. raw fruit containing 0·11 g. N) was included in the diet. The letter (O) indicates that this fruit was omitted.

For several days prior to Sept. 30, 1920 (period 1) a bread diet containing about 7·3 g. N had been taken in order to eliminate the disturbing effect of the previous high protein dietary. For the same reason the first three days of this period and the first five days of period 2 have been excluded in calculating the average nitrogen balance.

Table V.

Diet: whole wheat bread. Subject: R.R.

Period	Date 1920	Body weight *k*	Daily Intake: N bread g.	Daily Intake: N total g.	Daily Intake: Calories per kilo	Daily Output: N urine g.	Daily Output: N faeces g.	Daily Output: N total g.	Balance N g.
1. (A)	Sept. 30–Oct. 2	58·65 (27. ix)	5·55	5·82	48	—	—	—	—
(O)	Oct. 3–5	57·70 (6. ix)	„	5·71	49	5·62	1·47	7·09	−1·38
2. (A)	„ 10–14	58·20 (13. x)	8·32	8·57	54	—	—	—	—
	„ 15		„	„	„	7·21	1·98	9·19	−0·62
	„ 16		„	„	„	8·30	„	10·28	−1·71
	„ 17	58·13	„	„	„	6·41	„	8·39	+0·18
		Average	8·32	8·57	54	7·31	1·98	10·29	−0·72
3. (A)	„ 18		8·65	8·91	56	8·06	1·25	9·31	−0·40
	„ 19		„	„	„	8·70	„	9·95	−1·04
	„ 20		„	„	„	7·79	„	9·04	−0·13
		Average	8·65	8·91	56	8·18	1·25	9·43	−0·52
4. (A)	„ 21–23	57·89 (21. x)	9·73	10·00	58	8·32	1·70	10·02	−0·02
5. (O)	„ 24		9·73	9·84	58	8·41	1·81	10·22	−0·38
	„ 25		„	„	„	8·25	„	10·06	−0·22
	„ 26		„	„	„	10·51	„	12·32	−2·48
	„ 27	57·75	„	„	„	8·83	„	10·64	−0·80
	„ 28		„	„	„	7·67	„	9·48	+0·36
	„ 29		„	„	„	9·74	„	11·55	−1·71
	„ 30		„	„	„	9·50	„	11·31	−1·47
	„ 31	57·44	„	„	„	8·54	„	10·35	−0·51
		Average	9·73	9·84	58	8·93	1·81	10·74	−0·90
6. (O)	Nov. 1		11·89	11·98	63	9·30	2·68	11·98	0
	„ 2		„	„	„	9·75	„	12·43	−0·45
	„ 3		„	„	„	8·86	„	11·54	+0·44
	„ 4	57·70 (5. xi)	„	„	„	9·46	„	12·14	−0·16
		Average	11·89	11·98	63	9·34	2·68	12·02	−0·04

A marked rise in the nitrogen output occurred with both C.J.M. and R.R. at the beginning of period 5 and the coincidence of the omission of the apples that had been included in the diet during the preceding period led us to consider whether there was here any relation of cause and effect. Two possibilities suggest themselves: (1) the small amount of apple protein (0·11 g. N) might possess high value to supplement the wheat protein; (2) the alkaline

ash of the fruit would partially neutralise the acid ash of the bread and this might affect the nitrogen expenditure. Neither of the above appears adequate to explain the facts and further, the results obtained during period 4 do not agree any better with those for periods 2 and 3, in which apples were included in the diet, than with those for period 5 in which they were omitted. The increased output must therefore, like the variations which occur from day to day, be left for the present unexplained.

From the results of these experiments we have calculated the biological value of the wheat proteins by two formulae, which differ only in the assumption made with regard to the nitrogen of the faeces. Both are based on Thomas's definition of this value as the number of parts of body nitrogen spared by 100 parts of the nitrogen of the food, and, when reduced to their simplest form, can be thus expressed

$$\text{B.V.} = 100\,\frac{\text{Body N spared}}{\text{Food N absorbed}} = 100\,\frac{\text{Balance}\,[P] - \text{Balance}\,[M]}{\text{Intake}\,[P] - \text{Intake}\,[M]}$$

where P signifies the experiment with the protein under investigation and M the nitrogen minimum experiment.

This correction for the small amount of nitrogen in the diet of the N-minimum experiment is strictly accurate only if certain provisos hold, viz. (1) that the same amount of nitrogen in the same form, or of the same biological value, enters also into the second diet (P); (2) that the value of this nitrogen is not increased by supplementary action with the other proteins of the second diet.

In our experiments with wheat and milk proteins the first proviso is partly but not entirely satisfied. Whether or not the second is also satisfied cannot be decided. The method, however, certainly involves a less error than if the N-intake in the N-minimum experiment is altogether ignored.

When the different assumptions as to the faeces are made the two formulae become:

$$\text{I.}\quad \text{B.V.} = 100\,\frac{\text{Balance}\,[P] - \{\text{Intake}\,[M] - (\text{Urine N}\,[M] + \text{Faeces N}\,[P])\}}{\text{Intake}\,[P] - \text{Intake}\,[M]}.$$

$$\text{II.}\quad \text{B.V.} = 100\,\frac{\text{Balance}\,[P] - \text{Balance}\,[M]}{\text{Intake}\,[P] - (\text{Faeces N}\,[P] - \text{Faeces N}\,[M]) - \text{Intake}\,[M]}.$$

In I the whole of the food nitrogen is assumed to have been absorbed so that the total intake is also the real intake. In calculating the minimum expenditure corresponding with the period in question the faeces N for this period is added to the urine N $[M]$ and the intake $[M]$ subtracted from the sum. This formula corresponds with Thomas's formula B.

In II when the nitrogen of the faeces is in excess of that occurring in the N-minimum experiment this excess has been assumed to represent unabsorbed food and has been subtracted from the total intake to obtain the real intake. The minimum expenditure has been taken as the actual balance on N-minimum diet, *i.e.* Urine N $[M]$ + Faeces N $[M]$ − Intake $[M]$. This formula corresponds with Thomas's formula C except that in the latter an average

figure of 1 g. N, has been taken to represent the faeces N on a N-free diet. Both procedures have obvious disadvantages but there is very little difference in the results whichever is adopted.

A summary of the results for the separate periods with the biological values calculated from both the above formulae is given in Table VI.

Table VI.

Period	Intake: Total N g.	Intake: Absorbed N g.	Intake: Calories per kilo	Balance N g.	Biological value (1)	Biological value (2)
Diet: whole wheat bread. Subject: C.J.M.						
1. (O)	5·70	4·73	44	−2·06	38·8	25·3
2. (A)	8·63	7·63	56	−1·16	36·3	27·6
3. (A)	8·97	7·79	57	−0·80	41·1	31·8
4. (A)	9·98	9·41	53	+0·75	41·0	43·2
5. (O)	9·85	9·08	60	−0·51	36·0	30·4
6. (O)	12·00	10·64	61	+0·24	40·9	33·1
				Average	39·0	31·9
Diet: whole wheat bread. Subject: R.R.						
1. (O)	5·71	5·37	49	−1·38	34·2	29·8
2. (A)	8·57	7·72	54	−0·72	36·8	29·3
3. (A)	8·91	8·79	56	−0·52	28·7	27·9
4. (A)	10·00	9·43	58	−0·02	35·5	31·4
5. (O)	9·84	9·16	58	−0·90	28·0	22·5
6. (O)	11·98	10·40	63	−0·04	37·7	28·1
				Average	33·5	28·2

The Biological Value of the Proteins of Cow's Milk.

The first experiment with nitrogen in the form of milk protein followed immediately after the second period on low nitrogen diet and was carried out in duplicate on C.J.M. and R.R. The total nitrogen intake was approximately equal to the minimum nitrogen expenditure and though equilibrium was not obtained the negative balance was not very large, from which we concluded that milk proteins would be found to possess a relatively high value. When the experiments were repeated with larger amounts of milk we were surprised to find the nitrogen balance still negative and equilibrium was only reached with diets containing over 11 g. of milk nitrogen. This result appeared so extraordinary as to lead us to suspect that it might be due to a deficiency in the energy value of the diet, although this was amply sufficient to cover our normal requirements. The unusually rapid loss in weight which occurred in some of these experiments pointed in the same direction, as did also the coincidence of the fall in the nitrogen output occurring in one period (C.J.M. 6–R.R. 3) with a pronounced rise in the temperature of the air.

A further series of experiments was therefore carried out on one of us (C.J.M. periods 7, 8, 9), the fuel value of the diet being increased to the maximum that could be consumed. In the first of these, nitrogen equilibrium

was practically attained with a diet containing 6·84 g. N and furnishing 57 calories per kilo. After an interval of one day, on which the basal ration together with a very little milk (3·0 g. N) was consumed, period 8 was begun. The diet contained 5·28 g. N and furnished 55 calories per kilo, but the effects of the continued excessive diet made themselves unpleasantly obvious in the form of a bilious attack, which threatened to terminate the experiment. By reducing the amount of carbohydrate consumed, so that the fuel value fell to 36 calories per kilo it was however just possible to carry on and after two days the condition was so far improved that the full diet was resumed. The nitrogen intake was not altered at all but the effect of the reduced diet was very marked in the increased nitrogen output, which persisted for several days after the calories were again increased. The period was extended for six days after the output had reached a fairly constant level and only the results for these days have been considered in calculating the average output. A decided though small negative balance occurred.

Table VII.

Milk. C.J.M.

Foodstuff	Nitrogen per 100 g.	Calories per 100 g.	Diet during period 9 20–25. viii. 21 Weight g.	Nitrogen g.	Calories
Milk	0·508	65	830	4·216	539
Starch	0·027	360	280	0·076	1008
Lactose	0·013	370	210	0·027	777
Honey	0·023	327	250	0·057	818
Margarine (rendered)	0·010	900	18	0·002	162
Sucrose	—	395	10	—	40
Tea	0·008	—	750 cc.	0·060	—
Agar-agar	0·242	—	6	0·014	—
Salts	—	—	4	—	—
			Total	4·452	3344
			Excluding tea N	4·392	54 per kilo

In period 9 the quantity of milk was again reduced but the high calorie value of the diet was maintained. The usual daily game of tennis was discontinued and no exercise was taken so that the excess of energy supplied was even greater than before. The weather also was very hot. The average of the last six days of this period showed a considerable negative balance and the biological value calculated from this agrees fairly well with that obtained from the results of periods 7 and 8 and also of period 2 (C.J.M.). This point is interesting because the calorie value of the diet in period 2 was lower than in any other, only 44 per kilo. The composition of the diet during period 9 (C.J.M.) is given in Table VII and the results of the experiments are set out in Tables VIII (R.R.) and IX (C.J.M.), while Table X is a summary of these showing the biological values calculated from the two formulae. It is obvious from the amounts of nitrogen in the faeces that the milk proteins were very completely absorbed so that formula 1 probably gives the closest approximation to the truth in this case.

Table VIII.

Diet: milk. Subject: R.R.

Period	Date 1920	Body weight *k*	Daily Intake: N milk g.	Daily Intake: N total g.	Daily Intake: Calories per kilo	Daily Output: N urine g.	Daily Output: N faeces g.	Daily Output: N total g.	Balance N g.
"N-free"	Dec. 5	57·75	0	0·27	44	2·01	1·13	3·14	−2·87
1.	„ 6		2·73	2·98	48	2·49	0·66	3·15	−0·17
	„ 7		2·69	2·94	„	2·95	„	3·61	−0·67
	„ 8		2·76	3·09	„	3·22	„	3·88	−0·79
	„ 9		2·59	2·91	„	2·86	„	3·52	−0·61
	„ 10	57·50 (11. xii)	2·67	2·97	„	2·83	„	3·49	−0·52
	Average of last 4 days		2·68	2·98	48	2·97	0·66	3·63	−0·65
	1921								
2.	May 28	59·65	4·10	4·30	40	7·12	—	—	—
	„ 29		6·14	6·29	48	5·97	1·18	7·15	−0·86
	„ 30		„	„	„	5·91	„	7·09	−0·80
	„ 31		„	„	„	6·34	„	7·52	−1·23
	June 1		„	„	„	7·09	„	8·27	−1·98
	„ 2		„	„	„	6·55	„	7·73	−1·44
	„ 3	57·90 (4. vi)	„	„	„	6·21	„	7·39	−1·10
	Average of last 4 days		6·14	6·29	48	6·55	1·18	7·73	−1·44
	June 16			Mixed diet		12·29	—	—	—
3.	„ 17	59·60	13·61	13·69	47	13·48	1·37	14·85	−1·16
	„ 18		„	„	„	13·13	„	14·50	−0·81
	„ 19		„	„	„	12·29	„	13·66	+0·03
	„ 20		„	„	„	13·76	„	15·13	−1·44
	„ 21		„	„	„	12·39	„	13·76	−0·07
	„ 22		„	„	„	12·60	„	13·97	−0·28
	„ 23	58·50	„	„	„	11·10	„	12·47	+1·22
	„ 24		„	„	„	11·19	„	12·56	+1·13
	Average of last 5 days		13·61	13·69	47	12·21	1·37	13·58	+0·11

Table IX.

Diet: milk. Subject: C.J.M.

Period	Date 1920	Body weight *k*	Daily Intake: N milk g.	Daily Intake: N total g.	Daily Intake: Calories per kilo	Daily Output: N urine g.	Daily Output: N faeces g.	Daily Output: N total g.	Balance N g.
"N-free"	Dec. 5	60·45	0	0·33	48	2·13	1·17	3·30	−2·97
1.	„ 6		3·15	3·37	49	2·42	1·29	3·71	−0·34
	„ 7		„	3·36	„	2·57	„	3·86	−0·50
	„ 8		„	3·42	„	3·01	„	4·30	−0·88
	„ 9		„	3·40	„	3·12	„	4·41	−1·01
	„ 10	59·30 (11. xii)	„	3·45	„	3·22	„	4·51	−1·06
	Average of last 3 days		3·15	3·42	49	3·12	1·29	4·41	−0·99
	1921								
2.	April 30	62·60	4·02	4·17	44	4·74	1·22	5·97	−1·80
	May 1		„	„	„	4·58	„	5·80	−1·63
	„ 2		„	„	„	4·09	„	5·32	−1·15
	„ 3		„	„	„	4·25	„	5·47	−1·30
	„ 4	62·20	„	„	„	4·53	„	5·75	−1·58
	Average of last 4 days		4·02	4·17	44	4·36	1·22	5·58	−1·41
	May 10			Mixed diet		8·00	—	—	—
3.	„ 11	62·20	6·20	6·35	48	6·13	1·18	7·31	−0·96
	„ 12		„	„	„	5·71	„	6·89	−0·54
	„ 13		„	„	„	5·26	„	6·44	−0·09
	„ 14		„	„	„	5·80	„	6·98	−0·63
	„ 15		„	„	„	6·08	„	7·26	−0·91
	„ 16		„	„	„	6·07	„	7·25	−0·90
	„ 17	62·15 (18. v)	„	„	„	6·21	„	7·39	−1·04
	Average of last 4 days		6·20	6·35	48	6·04	1·18	7·22	−0·87

Table IX (*continued*)

Diet: milk. Subject: C.J.M.

Period	Date 1921	Body weight *k*	Daily Intake: N milk g.	Daily Intake: N total g.	Daily Intake: Calories per kilo	Daily Output: N urine g.	Daily Output: N faeces g.	Daily Output: N total g.	Balance N g.
"N-free"	May 27		—	—	—	8·38	—	—	—
4.	„ 28	62·40	7·63	7·78	47	7·05	0·96	8·01	−0·23
	„ 29		„	„	„	7·11	„	8·07	−0·29
	„ 30		„	„	„	7·55	„	8·51	−0·73
5.	„ 31		8·62	8·77	47	7·48	0·98	8·46	+0·31
	June 1		„	„	„	8·58	„	9·56	−0·79
	„ 2	61·30	„	„	„	8·41	„	9·39	−0·62
	„ 3		„	„	„	8·67	„	9·65	−0·88
	Average of last 3 days		8·62	8·77	47	8·55	0·98	9·53	−0·76
	June 16			Mixed diet		10·12	—	—	—
6.	„ 17	61·80	11·32	11·44	46	9·71	1·45	11·16	+0·28
	„ 18		„	„	„	10·46	„	11·91	−0·47
	„ 19		„	„	„	10·21	„	11·66	−0·22
	„ 20		„	„	„	10·19	„	11·64	−0·20
	„ 21		„	„	„	10·82	„	12·27	−0·83
	„ 22		„	„	„	8·09	„	9·54	+1·90
	„ 23		„	„	„	9·60	„	11·05	+0·39
	„ 24		„	„	„	9·71	„	11·16	+0·28
	„ 25	61·70 (26. vi)	„	„	„	10·28	„	11·73	−0·29
	Average of last 6 days		11·32	11·44	46	9·78	1·45	11·23	+0·21
	July 2			Mixed diet		6·33	—	—	—
7.	„ 3	61·10	6·65	6·84	57	5·66	1·28	6·94	−0·10
	„ 4		„	„	„	5·55	„	6·83	+0·01
	„ 5		„	„	„	5·35	„	6·63	+0·21
	„ 6		„	„	„	5·70	„	6·98	−0·14
	„ 7		„	„	„	6·35	„	7·63	−0·79
	„ 8	61·80	„	„	„	5·04	„	6·32	+0·52
	Average of last 4 days		6·65	6·84	57	5·61	1·28	6·89	−0·05
	July 9	62·10	—	3·00	54	5·16	—	—	—
8.	„ 10		5·16	5·28	55	4·54	1·09	5·63	−0·35
	„ 11		„	„	36	3·99	„	5·08	+0·20
	„ 12	61·00	„	„	36	5·57	„	6·66	−1·38
	„ 13	60·80	„	„	53	5·28	„	6·37	−1·09
	„ 14	61·10	„	„	57	5·29	„	6·38	−1·10
	„ 15	61·30	„	„	55	4·71	„	5·80	−0·52
	„ 16	61·60	„	„	54	4·63	„	5·72	−0·44
	„ 17	61·90	„	„	53	4·83	„	5·92	−0·64
	„ 18	61·85	„	„	52	4·06	„	5·15	+0·13
	„ 19	61·85	5·04	5·18	52	4·65	„	5·74	−0·56
	„ 20	62·00	„	„	52	4·24	„	5·33	−0·15
	Average of last 6 days		5·12	5·25	53	4·52	1·09	5·61	−0·36
9.	Aug. 16	61·60	4·24	4·58	53	15·79	—	—	—
	„ 17	61·85	„	„	„	10·78	—	—	—
	„ 18	61·85	„	„	„	6·46	—	—	—
	„ 19	61·90	„	„	„	5·83	—	—	—
	„ 20		„	4·39	„	4·69	1·00	5·69	−1·30
	„ 21	61·80	„	„	„	4·00	„	5·00	−0·61
	„ 22	61·80	„	„	„	4·10	„	5·10	−0·71
	„ 23	61·60	„	„	„	5·04	„	6·04	−1·65
	„ 24	61·60	„	„	58	4·78	„	5·78	−1·39
	„ 25	61·75	„	„	55	4·72	„	5·72	−1·33
	Average of last 6 days		4·24	4·39	55	4·56	1·00	5·56	−1·17

Table X.

Period	Intake: Total N g.	Intake: Absorbed N g.	Intake: Calories per kilo	Balance N g.	Biological value (1)	Biological value (2)
Diet: milk. Subject: C.J.M.						
1.	3·42	3·30	49	−0·99	74·7	73·6
2.	4·17	4·12	44	−1·41	47·3	46·6
3.	6·35	6·34	48	−0·87	38·4	38·3
5.	8·77	8·77	47	−0·76	26·3	28·6
6.	11·44	11·16	46	+0·21	33·0	31·2
7.	6·84	6·73	57	−0·05	49·7	48·8
8.	5·25	5·25	53	−0·36	55·6	57·2
9.	4·39	4·39	55	−1·17	45·2	49·4
			Average for last 3 periods		50·2	51·8
Diet: milk. Subject: R.R.						
1.	2·98	2·98	48	−0·65	66·0	83·6
2.	6·29	6·24	48	−1·44	25·0	24·4
3.	13·69	13·45	47	+0·11	24·2	22·9

Table XI. *Basal metabolism of C.J.M. on normal diet and on carbohydrate and fat (= 55 cals. per kilo) with 4·4 g. milk N.*

Date	Diet during previous 24 hrs.	Oxygen consumed per min. cc.	R.Q.	Calories per 24 hrs.
Aug. 10	Normal	225·6	0·711	1557
„ 12	„	236·3	0·928	1699
„ 13	„	227·1	0·684	1566
„ 14	„	220·9	0·820	1557
				Average 1595
„ 20	Milk	211·5	0·974	1529
„ 21	(4·39 g. N, 55 cals.	178·0	1·159	1359
„ 22	per kilo)	197·3	1·093	1478
„ 23	„	185·7	1·152	1415
„ 24	„	195·0	1·044	1440
„ 25	„	196·1	1·055	1453
„ 26	„	210·4	1·017	1540
				Average 1459
„ 27	Normal	221·9	0·915	1590
„ 28	„	216·8	0·889	1550
„ 31	„	228·0	0·811	1604
				Average 1581

During period 9 the subject's basal metabolism was determined on waking and the results were compared with similar determinations carried out during previous and succeeding periods when the diet was normal. These results are set out in Table XI and show a decrease of about 8 % in the basal metabolism on the milk diet.

DISCUSSION OF RESULTS.

The Minimum Nitrogen Expenditure.

During the second experiment to determine our nitrogen minimum the output of nitrogen in the urine fell to an amount equal to 0·035 g. per kilo body weight in the case of C.J.M. and 0·034 g. per kilo for R.R. The average amounts for the last three days of this period were slightly above the minima, being 0·038 g. and 0·035 g. respectively. If the nitrogen of the faeces is included the average output for the same period was 0·057 g. per kilo for C.J.M., and 0·055 g. for R.R. The nitrogen intake amounted to 0·005 g. per kilo.

These figures are somewhat lower than most of those recorded by other workers [Landergren 1903; Folin, 1905; Kinberg, 1911; Graham and Poulton, 1912], but this may be explained by the fact that their diets unavoidably contained more nitrogen than ours. Karl Thomas [1910] determined his minimum expenditure on a purely carbohydrate diet in seven experiments carried out over a period of two and a half years and found that this minimum fell from experiment to experiment, the final amount for the output in the urine alone being 2·2 g. or 0·029 g. per kilo, while that for urine and faeces combined was 2·9 g. or 0·039 g. per kilo. At this period Thomas weighed 75 kilos and had put on a good deal of body fat. In some of his earlier experiments, however, it seems probable that the minimum output was never reached as the diet was continued for too short a period.

In McCollum's [1911] experiments on pigs, which were fed on a diet of starch, a salt mixture and water, the minimum nitrogen output fell to a level corresponding closely with that reached by us. Thus a pig weighing 68·4 kilos excreted 0·039 g. N per kilo in the urine. For smaller animals the output per kilo was somewhat greater.

The determination of the nitrogen in urine and faeces does not, of course, give a complete account of the loss of nitrogen from the body. To these must be added the loss through hair, beard and nails, through loss of epidermis and in sweat. Except for the last named these losses are all very small in amount but the loss through the sweat may be considerable. Benedict [1906] has shown that a resting man may excrete 0·071 g. N per day in this way while with moderate work the loss may amount to 0·13 g. N per hour. McCollum considers that the nitrogen of the faeces should also be classed with these as representing losses that may be termed accidental in character and that the nitrogen of the urine alone is to be taken as representing the essential tissue metabolism of the body.

The amount of nitrogen excreted in the urine on the successive days of the experiments in which the diet was nearly free from nitrogen, is plotted in Figs. 2, 3, and 5. The amount diminishes in a fairly regular manner, at first quickly and then more slowly until the minimum is reached. The points lie on or near the graph of a simple logarithmic equation

$$\log (y - \lambda) = a - kx$$

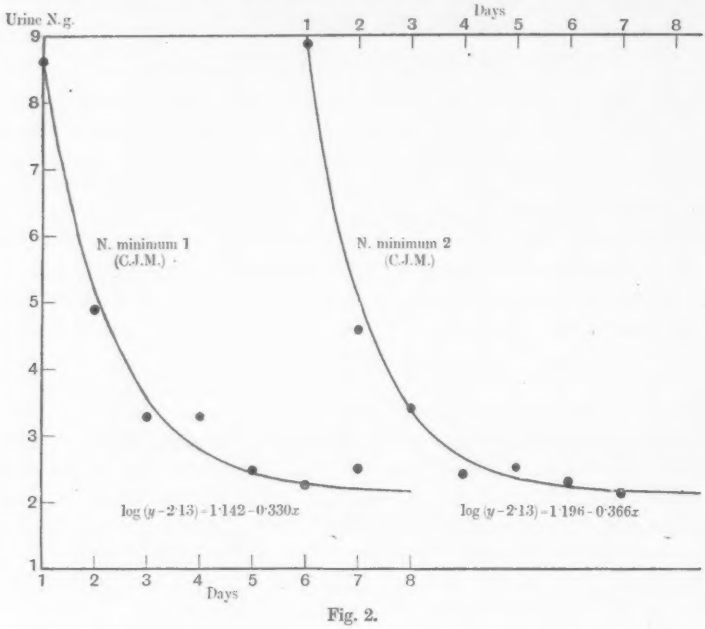
Urine N.g.
Days
N. minimum 1
(C.J.M.)
N. minimum 2
(C.J.M.)
log (y - 2·13) = 1·142 - 0·330x
log (y - 2·13) = 1·196 - 0·366x
Days

Fig. 2.

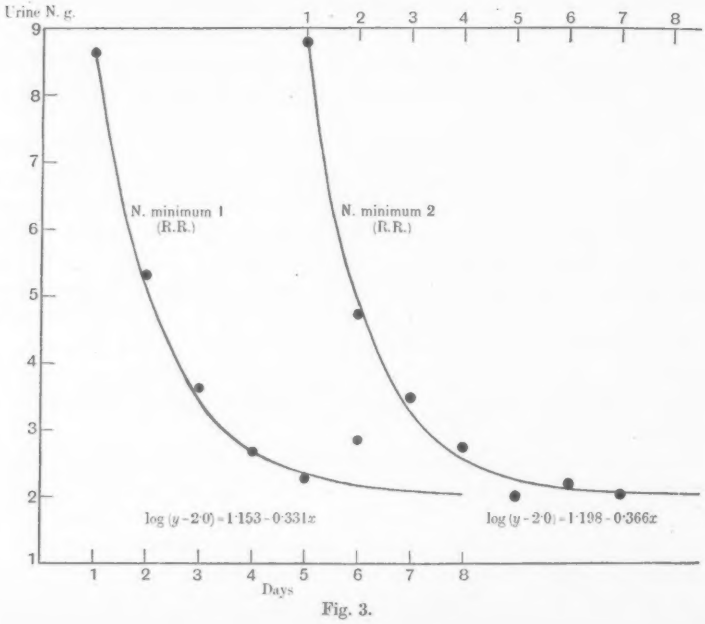
Urine N. g.
N. minimum 1
(R.R.)
N. minimum 2
(R.R.)
log (y - 2·0) = 1·153 - 0·331x
log (y - 2·0) = 1·198 - 0·366x
Days

Fig. 3.

in which x is the number of days, y is the nitrogen output in the urine, and λ the minimum value of y. A closer agreement is obtained if λ is given a value slightly lower than the minimum actually reached.

Thus in Fig. 3 the curve R.R. 1 is drawn from the equation

$$\log (y - 2{\cdot}0) = 1{\cdot}153 - 0{\cdot}331x$$

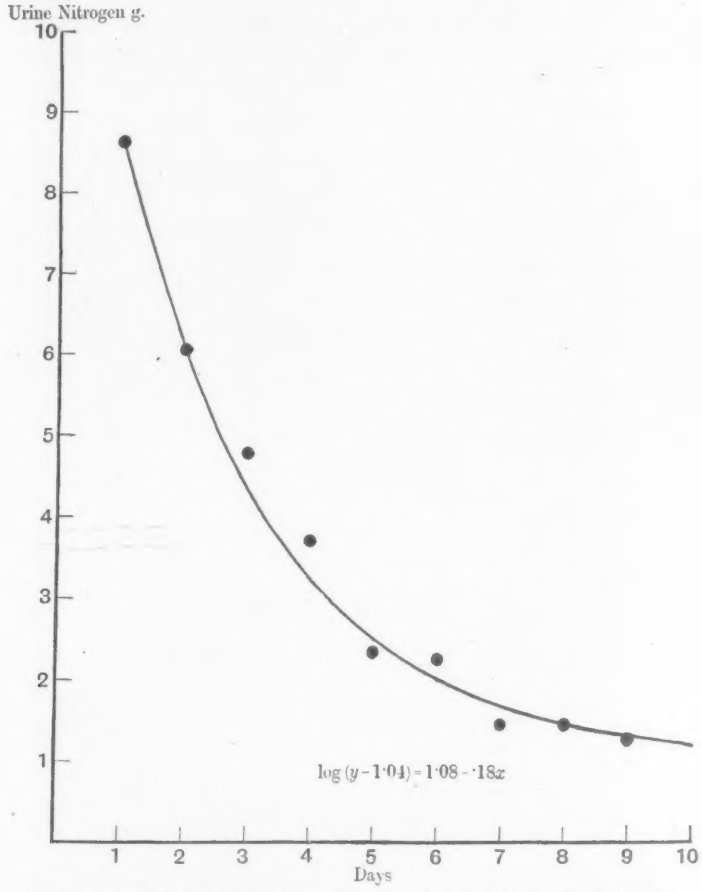

Fig. 4. E. V. McCollum's experiment on a pig. Daily output of nitrogen in urine on starch diet after ingestion of zein.

and fits the points reasonably well, but a still closer approximation is obtained with the equation $\log (y - 1{\cdot}73) = 1{\cdot}1209 - 0{\cdot}2832x$

the agreement between the observed and calculated values of y being extraordinarily good for all points except the last.

Day of experiment	y calculated from equation $\log(y-1{\cdot}73)=1{\cdot}1209-0{\cdot}2832x$	y obtained
1	8·61	8·64
2	5·31	5·31
3	3·60	3·63
4	2·70	2·66
5	2·24	2·25
6	1·99	2·82
20	1·73	—

A question is thus raised: Does this amount 1·73 g. represent the real minimum expenditure, which, from some cause was not realised in either experiment? At present this must be left unanswered, but further experiments may give some information on the point.

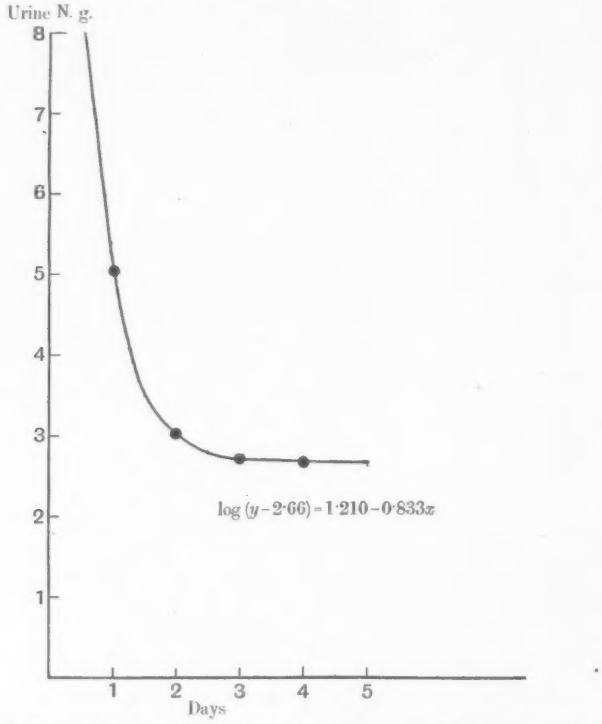

Fig. 5. E. V. McCollum's experiment on a pig. Daily output of nitrogen in urine on starch diet after ingestion of urea.

The difference between experiments 1 and 2 lies only in the amount of total fluid taken which was about 2000 cc. daily in the first and 4000 cc. in the second. This makes but slight difference in the value of k, that is in the rate at which the nitrogen output falls. Kinberg [1911] has previously drawn attention to this regularity but has not attempted to deduce any mathematical

expression from his results. Thomas [1910] also recognised that on a protein-free diet the nitrogen of the "Vorratseiweiss" leaves the body with varying rapidity according to the amount present, but was unable to find any exact relationship either in his own results or in those of Landergren. He calculated the amount of "Vorratseiweiss" excreted, by subtracting the minimum output ("Abnutzungsquota") from the urine nitrogen, but the value of this minimum was taken from experiments of four days' duration and was probably too high. Landergren's results show a fair agreement with the graph of an equation of the type given above if λ is taken as 2·5 instead of 3·0. Thomas's results are more irregular. In considering the agreement of the results of such experiments

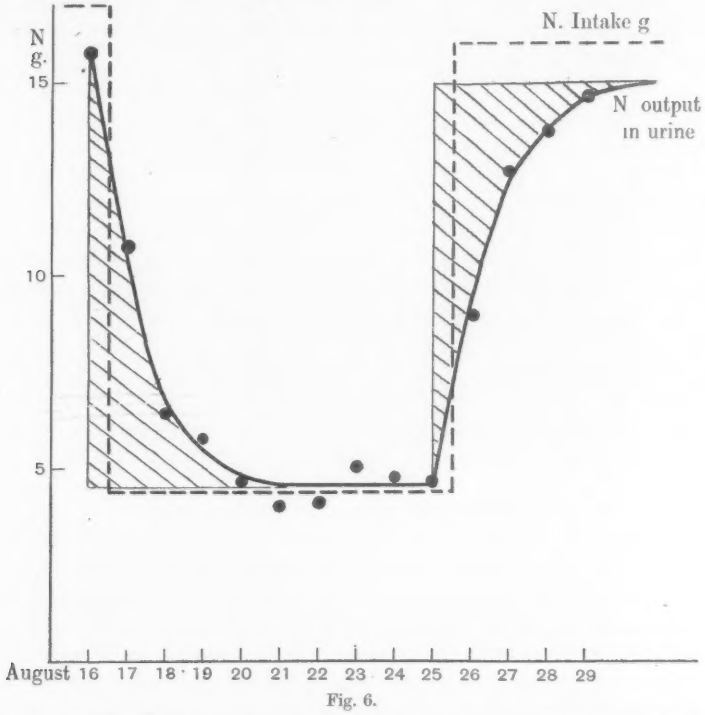

Fig. 6.

with those calculated from these equations, the tendency of the nitrogen output to oscillate even when the nitrogen intake is constant must be borne in mind. Such oscillations are present in most of the experiments considered, but do not alter the general character of the curve. In McCollum's experiments on pigs a similar regularity also appears as may be seen from Figs. 4 and 5. Fig. 4 shows the nitrogen output on a starch diet after a diet containing zein and Fig. 5 shows the output after the ingestion of urea. The curves are of the same type but "k," *i.e.* the rate at which the output falls, is very much

greater after urea than after zein. This point is of interest as it seems to indicate that the store of nitrogen which exists in the body after a protein diet, and which is rapidly given up on a diet free from nitrogen is not present entirely in the form of urea. As to the form in which this nitrogen occurs, very little can be definitely stated. It may be as resynthesised protein (Vorratseiweiss), or as amino acids adsorbed in the tissues, or compounds of intermediate complexity. It is certainly present partly as urea and other end products of metabolism. If our conclusions as to the rate at which this storage nitrogen is excreted are correct the interpretation is, that the amount removed from the body on any day is proportional to the amount still present. This might hold whether the reaction involved was the hydrolysis of protein, the deaminisation of amino acids or simply the washing out from the tissues of the end products of nitrogen metabolism.

On reversing the process, and after a minimal N-intake for ten days, suddenly increasing the nitrogenous food consumed, nitrogen at first remains in the body and equilibrium between intake and output does not occur for several days. Fig. 6 is a graph of the results of an experiment designed to show this. The broken line represents intake of N, the solid line output. The shaded area on the left hand represents the stored N gradually removed on dropping the intake from 17 g. to 4·4 g. and the right-hand area the amount again stored on resuming a diet containing 16 g. N. The curves are reciprocal and the two shaded areas approximately correspond.

The Nature of the Minimum Nitrogen Requirements of the Body.

The low level to which the nitrogen output falls on a protein free diet is evidence of the smallness of the body's daily requirements in this respect. We know from Folin's [1905] researches that the reduction in the nitrogen on such a diet occurs mainly at the expense of the urea fraction, the ammonia and uric acid being reduced to a relatively much smaller extent while the creatinine remains constant. These facts led Folin to conclude that protein metabolism is of two types, (1) "tissue" or "endogenous," which tends to be constant, and is represented largely by such products as creatinine, neutral sulphur, and to a less extent by uric acid, (2) "exogenous" which varies with the amount of protein consumed and is represented chiefly by urea. The nitrogen required for processes of the first type is alone essential, but Folin recognised that equilibrium at this low level may not be possible since a certain amount of protein may always fall prey to the exogenous metabolism. The distribution of the nitrogenous constituents of the urine was determined during our second minimum experiment and the milk diet immediately succeeding this.

The results [Robison, 1922, 2] are similar to those of Folin. The minimum nitrogen output was lower than any recorded by him, and the percentage of urea was correspondingly reduced, the minimum figure being 37·2 %, while the sum of the urea and ammonia amounted to 54·8 % of the total nitrogen.

The multifarious transactions involved in endogenous metabolism are not likely to be conducted with perfect economy. When much protein is hydrolysed and the products mobilised and used for the synthesis of proteins of another composition such as those of the blood or for the manufacture of thyroxin or adrenaline it is unlikely that the whole balance of unwanted amino acids escapes deaminisation and conversion. Leakage of this kind may account for no inconsiderable fraction of the minimum nitrogen expenditure and it is perhaps in this direction that, by adaptation, the body may effect some saving. The experiments of Thomas and Hindhede would appear to show that this does in fact occur.

The amount of carbohydrate eaten may also influence the degree of this waste since the process of deaminisation is reversible and will be affected by the rapidity with which the non-nitrogenous products, hydroxy or ketonic acids, are removed by oxidation or conversion into carbohydrate. It is probable that an excess of carbohydrate in the blood would retard either action and in consequence deaminisation.

The Question of Uniformity of the Biological Value.

We have seen that the validity of the method adopted by Thomas for the determination of the biological values of proteins depends in the first place on the uniformity of this value when varying amounts of the same protein are consumed. The investigation of this question was one of the objects of our experiments and the results must now be considered from this point of view. The three proteins so far studied were chosen on account of the wide difference in the values attributed to them. The biological values of milk and wheat proteins as given by Thomas are 99·71 and 39·56 respectively, while gelatin has long been known to be deficient in several essential amino acids, so that no amount, however great, can completely satisfy the body's nitrogen requirements.

The results of the experiments described in this paper together with those on gelatin, previously recorded by one of us [Robison, 1922, 1] are plotted in Figs. 7 and 8, in which the values of the nitrogen intake for different periods are the abscissae and the corresponding amounts for the total output in urine and faeces are the ordinates. For this purpose the nitrogen of the faeces in excess of the amount on the "protein-free" diet has been taken as representing unabsorbed food and has been subtracted from both intake and output.

In the case of whole wheat proteins, the results are strikingly similar with both individuals and the points obviously lie on or close to a straight line passing through the points representing the minimum expenditure on the low nitrogen diet. The agreement is remarkably good considering the errors inseparable from such experiments. The results for periods 4 and 5 show some discrepancy but these periods were continuous and should probably be considered as a whole, the only difference in the diets being the inclusion of

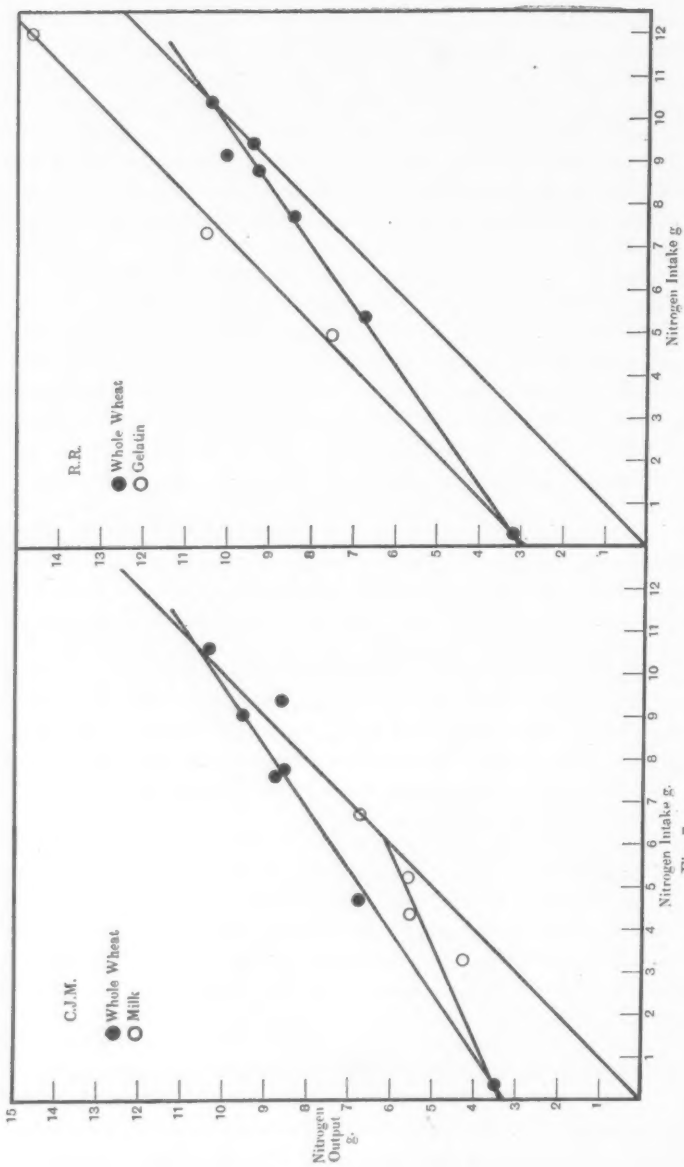
C.J.M.
Whole Wheat
Milk
Nitrogen Output g.
Nitrogen Intake g.
R.R.
Whole Wheat
Gelatin
Nitrogen Intake g

Fig. 7.

Fig.8.

a small quantity of apples in the former and its omission from the latter. The average for the two periods gives a point lying close to the line. The uniformity of the biological value is therefore satisfactorily proved for the proteins of whole wheat.

In the case of gelatin also the points fit a straight line reasonably well, but if this line passes through the point representing the nitrogen minimum (as drawn in the figure), it will at some distant point intersect the "equilibrium line" (at 45° to the axis), *i.e.* nitrogenous equilibrium will be attained at this point, which we believe to be impossible. On the other hand if it is drawn parallel to the "equilibrium line" it will cut the axis of y at a point slightly below the observed minimum. We must conclude, therefore, either that the line is slightly curved near the minimum or that the observed value of the latter is somewhat higher than the real one. If the capacity of the gelatin is limited to the reduction of "leakage" these two alternatives have practically the same significance.

Milk presents a more difficult case. Only the results for those periods in which a diet of abundant fuel value was taken (C.J.M. 1, 7, 8, 9) have been plotted but even these do not agree well with any straight line or other regular curve. The most that can be said is that in view of the possible errors in such experiments where the intake is small, the results are not inconsistent with the uniformity of the biological value of this protein.

From the consideration of these three cases we may conclude that the general assumption of this principle made by Thomas, occasioned no serious errors in his conclusions. Caution must be used in extending this principle to all cases without investigation and reliance should not be placed upon results of experiments in which the negative balance is large.

The Biological Values of the Proteins of Whole Wheat and Milk.

Whole Wheat.

The biological values calculated from the different periods of our experiments with whole wheat proteins agree very well amongst themselves and give an average of 35 for those obtained on C.J.M. and 31 for those obtained on R.R. Thomas's value is somewhat higher, 39·56. The latter figure was based on the result of two experiments each of three days' duration and one of four days. During none of these experiments was the nitrogen intake constant; in one, the amounts for the separate days were 4·0 g., 7·3 g., 9·0 g. respectively. The minimum requirements corresponding with these three periods were taken as 4·63 g., 3·991 g. and 3·316 g. N (urine only), these amounts being determined in experiments of four and three days' duration. In the first of these the nitrogen output on the four days was 18·32 g., 10·17 g., 7·39 g., 4·63 g. but there is no evidence that the last figure represented Thomas's minimum requirements. The biological value of wheat proteins was calculated from each separate

day's balance and out of these widely varying results those for certain days were selected in a somewhat arbitrary fashion. The experimental results of his third period, in which the intake was nearly constant, indicate a similar biological value for wheat proteins to that found by ourselves.

The result Hindhede [1913, 1] obtained upon F. Madsen with white bread, namely a positive balance of about 1 g. per day over a 28 day experiment on 13·73 g. N was certainly not a minimum quantity and the amount necessary for N equilibrium may be considerably less. His further experiments [1914] were made with rye bread ("Schwarzbrot") and the diets contained considerable amounts of fruit or vegetables, which accounted for 0·5 g. to 1·9 g. N. These experiments were carried out in duplicate on F. and H. Madsen and were continued for four months so that they possess great value. In the final period of six days the fruit was omitted and positive balances were obtained on 13·52 g. (F.M.) and 10·68 g. (H.M.) bread nitrogen respectively. By subtracting the whole of the nitrogen in the faeces Hindhede calculates that the amounts absorbed were 8·49 g. and 6·28 g. respectively. We do not agree with this method of treating the faecal nitrogen and cannot accept his conclusion that bread proteins possess equal value with those of potatoes, of meat and of the body; but there is no doubt that in his experiments, equilibrium was obtained on a lower intake of nitrogen in the form of rye bread (and still lower when supplemented by fruit) than our minimum for whole wheat. This is confirmed by the experiments of Neumann [1919], who, by long continued diet of very high calorie value, was ultimately able to retain nearly 3 g. N daily with an intake of 9·9 g. N in the form of rye proteins. Neumann's experiment was upon himself, and was in every way unexceptionable, but it may perhaps be significant that it was preceded by a prolonged period of semistarvation during which he was investigating the German civilian ration of 1916–17. This is a further indication that, apart from the influence of loss of body weight, the organism can gradually accommodate itself to a lower ration of nitrogen, perhaps by the exercise of greater economy.

Abderhalden's [1915] observations on Röse, although as pointed out earlier in this paper, not susceptible of the interpretation he places upon them, do indicate that the latter could get into equilibrium with about a gram less N in the form of white bread than we could with bread made from the whole grain. Röse's nitrogen expenditure on a nitrogen-free diet was not ascertained so we cannot estimate the biological value for this diet.

The recent observations of Rubner [1919] upon the proteins of white flour are interesting in relation to our own, because they show, we think, that from the point of view of biological value, the proteins of the endosperm are equal, if not superior to, those of the whole seed.

In most of Sherman's experiments on the value of cereal proteins these are supplemented by a certain amount of milk and the results are not directly comparable with ours, but in some experiments on white bread [1920] the diet consumed would not appear to be greatly different from that taken by

us and we are therefore the less able to explain the difference between his results and ours. Sherman's subject, a man weighing 80 kilos, attained equilibrium on a diet containing 6·0 g. N over 95 % of which was derived from white bread and the remainder from apples and butter. The energy value was only 34 calories per kilo. The bread was purchased from a bakery and probably contained a small amount of milk but how much was not known.

Milk.

The results of our experiments with milk proteins do not agree so closely as those for the whole wheat. The very low values calculated from the results of periods 3, 5, 6 (C.J.M.) and 2, 3, (R.R.) are not easy to explain. That they are in some way due to the lower calorie value of the diet seems clear but this was in no case below that of our normal diet and amply covered our energy requirements. The high values obtained with both subjects in period 1 are perhaps connected with the previous nitrogen starvation and a consequent increase in the economy with which the body may deal with the protein supplied to it.

If we consider only periods 7, 8, 9 (C.J.M.) in which the conditions were the same and the energy supply abundant the biological value for milk proteins is equal to 51 %. This value is only half of that found by Thomas, but the criticisms we have made in discussing his experiments with bread apply with still greater force in this case. His value (100 %) was calculated from the nitrogen balance on a single day of an experiment lasting only two days on which the intake was 6·24 g. and 7·28 g. respectively. The minimum requirements were taken as 3·99 g. which is probably much too high. If the value of milk protein were as high as Thomas makes out it is difficult to see how he could explain the large negative balances occurring in his experiment with "Frauenmilch" (cow's milk with extra sugar and cream). During the first two days of this experiment the fuel value of his diet was obviously too low, but in the last three days it was equal to 40–45 calories per kilo, the N-intake being 15·3 g.–17·3 g. yet the negative balance was never less than 1·0 g. The results of this experiment appear to agree with our experience, and to suggest that a high intake of milk nitrogen tends to result in increased expenditure of body nitrogen, unless the fuel value of the diet is raised very much above the normal. So far as we are aware the value of milk protein has not been the subject of any other investigation on man.

Conclusions.

From our observations upon ourselves we conclude:

(1) That our minimum nitrogen expenditure by the urine is somewhat less than 0·038 g. and 0·035 g. per kilo in C.J.M. and R.R. respectively.

(2) That on taking a diet of carbohydrate and fat of adequate calorie-value the nitrogen excreted in the urine falls in a regular and orderly manner,

capable of simple mathematical expression, approaching a minimum in five to seven days. On resuming an ordinary nitrogenous diet the reciprocal phenomenon occurs.

(3) Bearing in mind the considerable experimental errors, the ratio $\frac{\text{Body N saved}}{\text{Food N absorbed}}$ appears to remain constant. whatever amount of nitrogen is taken in the form of whole wheat bread, until equilibrium is reached.

(4) In the case of milk the experimental errors are proportionately greater and the most we can say is that this ratio may remain constant.

(5) In the case of gelatin the ratio certainly does not remain constant and there is no indication that the amount of body nitrogen saved increases beyond that effected by the smallest quantity of gelatin fed.

(6) The application of Thomas's method of determining biological values is justified in the case of bread, doubtful with milk and impossible with gelatin.

(7) Until Thomas's procedure has been ascertained to be justifiable for the particular proteins concerned, the ratio $\frac{\text{Body N saved}}{\text{Food N absorbed}}$ should be determined close to, but below, the point of equilibrium.

(8) The mean biological value of the nitrogen contained in the whole wheat grain as determined by six experiments on each of two adults was 35 % (C.J.M.) and 31 % (R.R.).

(9) The mean biological value of the nitrogen in cow's milk, derived from three experiments upon C.J.M. in which an excess of calories (55 per kilo) was taken, was 51 %.

(10) Biological values arrived at from experiments of comparatively short duration, however well justified, have a limited significance.

REFERENCES.

Abderhalden and Rona (1904). *Zeitsch. physiol. Chem.* **42**, 528.
—— —— (1906). *Zeitsch. physiol. Chem.* **47**, 397.
Abderhalden and Samuely (1905). *Zeitsch. physiol. Chem.* **46**, 193.
Abderhalden, Fodor and Röse (1915). *Pflüger's Archiv.* **160**, 511.
Albu (1901). *Zeitsch. klin. Med.* **43**, 75.
Atwater and Benedict (1902). *Mem. Nat. Acad. Science*, **8**, 233.
Benedict (1906). *J. Biol. Chem.* **1**, 263.
Bornstein (1898). *Berl. klin. Woch. Nr.* 36.
Boruttau (1915). *Biochem. Zeitsch.* **69**, 225.
—— (1919). *Biochem. Zeitsch.* **94**, 194.
Chittenden (1904). Physiological Economy in Nutrition, New York.
Cohnheim (1901). *Zeitsch. physiol. Chem.* **33**, 451.
—— (1906). *Zeitsch. physiol. Chem.* **49**, 64.
Falta (1906). *Arch. klin. Med.* **86**, 517.
Folin (1905). *Amer. J. Physiol.* **13**, 66 and 117.
Goldberger, Waring and Willets (1915). *U.S., P.H. Reports.* Reprint **307**, 5.
Goldberger, Wheeler and Sydenstricker (1920). *U.S., P.H. Service Reports*, **35**, 648.
Graham and Poulton (1912). *Quart. J. Med.* **6**, 82.
Grubner (1901). *Zeitsch. Biol.* **42**, 407.

Hindhede (1913, 1). *Skand. Arch. Physiol.* **28**, 165.
—— (1913, 2). *Skand. Arch. Physiol.* **30**, 97.
—— (1914). *Skand. Arch. Physiol.* **31**, 259.
Hirschfeld (1887). *Pflüger's Archiv.* **41**, 533.
—— (1889). *Virch. Archiv.* **114**, 301.
Hopkins and Cole (1901). *J. Physiol.* **27**, 418.
Kinberg (1911). *Skand. Arch. Physiol.* **25**, 291.
Klemperer (1889). *Zeitsch. klin. Med.* **16**, 550.
Kossel (1901). *Ber.* **34**, 3214.
Kossel and Kutscher (1900). *Zeitsch. physiol. Chem.* **31**, 165.
Kumagawa (1889). *Virch. Archiv.* **116**, 370.
Kutscher and Seemann (1901). *Zeitsch. physiol. Chem.* **34**, 527
Landergren (1903). *Skand. Arch. Physiol.* **14**, 112.
Lapicque (1894). *Arch. Physiol. Norm. et Path.* **26**, 596.
Lapicque and Marrette (1894). *C.R. Soc. Biol.* 10 ser. 1, 274.
Loewi (1902). *Arch. Exp. Path. Pharm.* **48**, 303.
McCollum (1911). *Amer. J. Physiol.* **29**, 210.
McCollum and Hoagland (1913). *J. Biol. Chem.* **16**, 317.
McCollum, Simmonds and Pitz (1917). *J. Biol. Chem.* **29**, 341.
McCollum, Simmonds and Parsons (1921). *J. Biol. Chem.* **47**, 111, 139, 175, 207, 235.
Michaud (1909). *Zeitsch. physiol. Chem.* **59**, 405.
Neumann (1899). *Arch. Hyg.* **36**, 248.
—— (1902). *Arch. Hyg.* **45**, 1.
—— (1919). *Vierteljahrschrift gericht. Med.* 52.
Osborne and Harris (1903). *J. Amer. Chem. Soc.* **25**, 853.
Osborne and Mendel (1911). Pub. no. 156. Carnegie Inst. Washington.
—— —— (1912–1920). *J. Biol. Chem.* **12** to **45**.
—— —— (1917). *J. Biol. Chem.* **29**, 69.
Peschel (1891). *Inaug. Dissert.* Berlin.
Robison (1922, 1). *Biochem. J.* **16**, 111.
—— (1922, 2). *Biochem. J.* **16**, 131.
Rubner (1879). *Zeitsch. Biol.* **15**, 115.
—— (1897). V. Leyden "Handbuch der Ernährungstherapie."
—— (1915). *Arch. Physiol.* 145.
—— (1919). *Arch. Physiol.* 81.
Sherman (1920). *J. Biol. Chem.* **41**, 97.
Sherman, Wheeler and Yates (1918, 1). *J. Biol. Chem.* **34**, 383.
Sherman and Winters (1918, 2). *J. Biol. Chem.* **35**, 301.
Sherman, Winters and Phillips (1919). *J. Biol. Chem.* **39**, 53.
Sivén (1900). *Skand. Arch. Physiol.* **10**, 91.
Thomas (1909). *Arch. Physiol.* 219.
—— (1910). *Arch. Physiol.* Suppl. 249.
Voit, C. (1866). *Zeitsch. Biol.* **2**, 307.
—— (1867). *Zeitsch. Biol.* **3**, 1.
Voit, E. and Korkunoff (1895). *Zeitsch. Biol.* **32**, 58.
Willcock and Hopkins (1907). *J. Physiol.* **35**, 88.
Wilson (1921). *J. Hygiene*, **20**, 1.
Zeller (1914). *Arch. Physiol.* 213.
Zisterer (1910). *Zeitsch. Biol.* **53**, 157.